Bluetongue

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Preface

Bluetongue, initially recognised in South Africa in late 1870s in sheep, is now known to affect a wide variety of other domestic and wild ruminants as well. Recent reports also suggested that camelids may also be affected with bluetongue virus in certain situations. Though it was recognised more than a century ago, it continues to be an economically very important livestock disease throughout the world. Since bluetongue is transmitted by *Culicoides* midges, its geographical distribution is dependent on the presence of the competent insect vector and susceptible vertebrate host. Thus occurrence of bluetongue virus infection in different parts of the world is governed by several ecological and environmental factors. We think that there has been unprecedented progress with greater impact offering clearer insight into knowledge about bluetongue virus in the past 20 years than there had been in the entire 40 years since bluetongue made its way to the OIE 'List A' disease. During this period, the discipline of molecular biology has profoundly influenced virtually every facet of arbovirology. The progress has been astounding and success phenomenal. The developments in molecular and immunological technologies, have given a great insight into the virus biology, genetic and antigenic diversity, host immune response, vector biology and vaccines. Enormous literature has accumulated due to extensive studies conducted on various basic and applied aspects of this important animal disease. However, there have been very limited efforts to compile the new research findings in comprehensive and understandable manner in the form of a book.

Bluetongue is the result of realisation of this need of having a book which documents all the relevant information in the past, present and future perspectives. Since there is no book exclusively devoted to this disease with Indian perspective, we believe that a comprehensive book on bluetongue dealing with all the aspects would be very useful to a broad group of individuals directly or indirectly involved with animal husbandry, trade on animals and their germplasm, wildlife conservation, researchers, teachers, under graduate and post graduate students, animal health professionals, policy makers in India as well as other Asian and African countries where bluetongue is endemic. The book has been planned in such a way that it would include from the very basic origin and history of bluetongue disease to the state-of-the-art information available on this economically important viral disease. Because of this nature of the book, it would have very vast readership in India and abroad.

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Abbreviations

AA	Amino acids
AGID	Agar gel immunodiffusion
AGPT	Agar gel precipitation test
AHC	Animal health certificate
AHS	African horse sickness
AHSV	African horse sickness virus
AI	Artificial insemination
BEI	Binary ethylenimine
B. ELISA	Blocking ELISA
BHK-21	Baby hamster kidney cell line
BPL	Beta propiolactone
BT	Bluetongue
BTV	Bluetongue virus
cDNA	Complementary DNA
cELISA	Competitive ELISA
CF	Complement fixation
CLP	Core-like particles
CMI	Cell-mediated immunity
CNS	Central nervous system
CPAE	Cattle pulmonary artery endothelian cell line
CPE	Cytopathic effect
CsCl	Cesium chloride
CSBF	Central Sheep Breeding Farm
CSWRI	Central Sheep and Wool Research Institute
CTL	Cytotoxic T-cells
DB	Dissemination barrier
DIA	Dot immunobinding assay
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DPC	Days post challenge
DPV	Days post vaccination
ds	Double stranded
ECE	Embryonating chicken eggs
EHD	Epizootic haemorrhagic disease
EHDV	Epizootic haemorrhagic disease virus
ELISA	Enzyme-linked immunosorbent assay
ELOSA	Enzyme-linked oligonucleotide-sorbent assay
FAT	Fluorescence antibody technique
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
FMD	Foot-and-mouth disease
HRPO	Horse radish peroxidase

I ELISA	Indirect ELISA
IETS	International embryo transfer Society
IF	Immunofluorescence
IFAT	Indirect fluorescent antibody test
KDa	Kilo Dalton
KVI	Kimron Veterinary Institute
MAb	Monoclonal antibody
MEB	Mesenteron escape barrier
MIB	Mesenteron infection barrier
MOI	Multiplicity of infection
mRAD	Milli radiation
MPH	Miles per hour
mRNA	Messenger RNA
NA	Nucleic acid
NCM	Nitrocellulose membrane
NR	Not reported
NS protein	Non-structural protein
OD	Optical density
OIE	Office of International Epizootics
OPG	Potassium oxalate (5g), phenol (5g), glycerine (500 ml)
PAGE	Polyacrylamide gel electrophoresis
PCV	Packed cell volume
PCR	Polymerase chain reaction
PFU	Plaque forming units
PI	Post-infection
PRA	Plaque reduction assay
RBC	Red blood corpuscles
RNA	Ribonucleic acid
RT	Reverse transcriptase
S	Svedberg
SAT	Saliva activated transmission
SEM	Scanning electron microscopy
SGIB	Salivary gland infection barrier
SGEB	Salivary gland escape barrier
SN	Serum neutralization
ss	Single stranded
$TCID_{50}$	Tissue culture infective $dose_{50}$
TEM	Transmission electron microscopy
TOTB	Trans-ovarian transmission barrier
VIBs	Virus inclusion bodies
VLP	Virus-like particles
VNT	Virus neutralization test
VP	Viral protein
WHO	World Health Organization

Contents

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Introduction

Bluetongue (BT), though recognized more than a century ago in South Africa, continues to be an economically very important disease affecting susceptible domestic and wild ruminants in tropical, semi-tropical and temperate regions of the world. Global economic importance of BT is evident from its OIE 'List A' disease status, and because of its status, mandatory restrictions are imposed on movement of ruminant animals, their germplasm, embryos and other animal products from BT endemic regions to BT free zones. Economic losses due to bluetongue virus (BTV) infection are mainly attributed to high morbidity, mortality, abortions, stillbirths, foetal abnormalities, meat and fleece loss.

Bluetongue viruses infect a wide variety of domestic and wild ruminants through insect vectors. Some vertebrate hosts are more susceptible as compared to the others. Sheep is the most susceptible for clinical disease, though there may be variation in susceptibility amongst different breeds of sheep. Cattle, buffalo, goats and several species of wild ruminants serve as reservoir host. BTV has also been recognized as a potential pathogen of carnivores. Inadvertent infection of pregnant bitches with BTV-contaminated vaccine has caused abortions and death. Serologic evidence of BTV infection has been demonstrated in several species of African wild carnivores. This clearly demonstrates that host range of BTV is much larger than previously believed.

Exact estimate of economic losses due to reproductive disorders caused by BTV in different species of ruminant livestock is not known. However, it is suggested that the virus infection may cause significant reproductive losses in cattle and sheep. The BTV infection may cause abortion and teratogenic effects in sheep and cattle under natural and experimental conditions. However, consequence of vertical transmission in sheep and cattle are variable. Factors influencing reproductive consequence are the stage of gestation, virulence of the virus, source and concentration of the virus inoculum, placentation, season and route of infection. Reproductive consequences of BTV infection vary in degree, in general includes infertility, abortion, and mummification of foetus, stillbirths, congenital abnormalities and dysfunction in live offsprings. Immunologic unresponsiveness, sporadic viraemia, development of late disease may be consequences of vertical transmission in offsprings of infected cattle. Perpetuation of BTV in three generations of cattle has been documented. Attenuated BTV appears to have

more affinity to placenta and causes foetal infection in vaccinated animals clearly demonstrating the reproductive impact of BTV infection in cattle herds and sheep flocks. Understanding of the mechanism by which the virus crosses placenta might help in controlling reproductive losses due to BTV. The presence of BTV in semen has also been demonstrated.

There are currently 24 serotypes of BTV, which are transmitted by several species of *Culicoides* vectors to diverse spectrum of vertebrate hosts. A number of variables are associated with the virus (serotype/strain/topotype/genotype/nucleotype), vectors, ecology and mammalian host, influencing the occurrence of BTV infection. Variations in the virulence of different serotypes/strains of BTV have been observed in sheep, and may be true to other susceptible species of ruminants. The studies have clearly indicated that all individuals of the field population of a *Culicoides* vector species may not be competent to transmit the disease. Ecological factors such as temperature, humidity, rainfall and vegetation, also affect the dynamics of the vector population. Studies on vector biology have suggested that vector capacity and the vector competence are governed by gene(s), which limit replication of the virus in different organs of the vector using different types of escape barrier systems. Therefore, understanding of interplay of different factors linked to vector-virus-host and environment leading to occurrence of BTV infection in a geographical region is crucial for developing effective control strategies.

Apparent failures of vaccines more than 50 years ago in South Africa in initial stages of development led to the realisation that there were more than one antigenic types of BTV. The classical cross protection experiments demonstrated that there was solid protection against homologous strain whereas only partial or no protection against heterologous strains. These strains were called 'serotypes'. Subsequently, recognising the global importance of the disease, Bluetongue World Reference Center was established at the Veterinary Research Institute in Onderstepoort, South Africa. It is now well established that bluetongue viruses of the same or different serotypes can exchange genetic information by the process of genetic reassortment during the mixed infection of the vertebrate host or insect vector. Advances in molecular biology has developed new parameters for measuring genetic diversity that would lead to better understanding of regional evolution of the viruses. However, the classical method of serotyping based on neutralisation, continue to be internationally accepted.

India is a geographically vast country having diverse geo-climatic zones. The country has huge population of domestic and wild ruminants, which are susceptible to BTV infection. Over the past three decades, several exotic breeds of sheep and cattle have been introduced in the country for improvement of indigenous stock through crossbreeding. Therefore, more susceptible animals for BTV infection are present now than in early 1960s when several outbreaks of BT occurred in indigenous and exotic sheep in Maharashtra (Sapre 1964) for the first time. Since then outbreaks of BT have been reported from several other states and 21 out of the 24 known

serotypes of BTV have been reported to be present in India (Prasad 2000). This emphasises the importance of BT in a developing country like India with a vast and promising domestic and wild ruminant population.

Protective immunisation against BTV has been a difficult goal for past several decades. The antigenic diversity exhibited by the known 24 serotypes of BTV and insect vector-borne nature of the infection have been the major impediments towards development of an effective broad based vaccine and control of the disease. The traditional methods for development of an effective BTV vaccine have been used to produce attenuated and inactivated vaccines in South Africa and in some other countries. However, similar vaccines based on the local Indian isolates are yet to be developed. Molecular techniques have been used to develop effective recombinant vaccines. Virus-like particles and core-like particles produced by baculovirus expression system have shown limited utility as practical vaccines due to reasons described elsewhere in the book.

Bluetongue virus belongs to *Orbivirus* genus of the family Reoviridae. The virus is architecturally complex and has ability to attach and replicate in a variety of vertebrate and invertebrate cells. It is composed of 10 discrete segments of dsRNA genome surrounded by two layers of protein capsid. In the past couple of years great insight has been gained into molecular structure of the virus. X ray crystallographic technique revealed the atomic structure of BTV core. Organisation of 10 segments of dsRNA has also been studied using crystallographic technique. These studies have helped in understanding the mechanism of the core particle assembly and packaging of genomic segments. However, it is still not clearly known how single copy of each of the 10 different viral genome segments is packaged inside the core particle? Though it can be speculated how the 24 serotypes might have emerged from a common or a few different ancestors, further studies are required to understand the time frame and the host, vector and the ecological conditions which might give rise to a new serotype.

Due to segmented dsRNA genome, the virus is more prone to frequent mutations leading to emergence of new genetic variants in the natural mammalian host and in the insect vector cycle. Therefore, it is necessary to develop methods suitable for group and serotype specific diagnosis as well as detection of genetic and antigenic diversity of the virus. In the last over two decades, a wide array of traditional as well as molecular approaches have been developed for diverse situations to detect the presence of BTV infection either by detecting the antibodies and antigens or the viral nucleic acids in the biological samples. The frank clinical disease is usually diagnosed on the basis of clinical signs characterised by pyrexia, swelling of muzzle, oral lesions, coronitis, stiffness of limbs, and in some cases by oedema of head and neck. However, diagnosis of sub-clinical and inapparent infection of BTV presents a difficult situation. Therefore, highly sensitive and specific methods are required to detect the virus infection.

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2

History

The time and place of the exact origin of BT remains largely speculative. However, it is believed that the disease was first noticed by farmers when highly susceptible Merino and other European breeds of sheep were introduced in southern Africa in late 1870s. Hutcheon who called it as 'epizootic catarrh' of South Africa first described the disease in 1881. The initial observation of the affected sheep revealed presence of cyanotic (bluish in colour) tongue, though it is now well known that all the clinically affected sheep do not exhibit cyanotic tongue. Since this disease was new or became apparent due to introduction of highly susceptible sheep, it caused serious concern amongst farmers and veterinarians. In 1892, Iwanowsky for the first time demonstrated that mosaic disease of tobacco plants was caused by a virus. Later, in 1897, Loeffler and Frosch reported that foot-and-mouth disease of animals was caused by a virus for the first time. Subsequently, the filterability of tobacco mosaic virus was confirmed by Beijerinick in 1899. Thus, plant disease causing agent which was filterable and much smaller than bacteria was discovered and this laid the foundation of new class of pathogens called viruses. The word, virus is derived from a Latin term meaning liquid poison.

With the descriptions of viral aetiology of foot-and-mouth disease, hunt started for the animal diseases which were due to non-bacterial agents. Since aetiology of BT was not known at that time, Spreull (1902) speculated that it was caused by an intracellular parasite or plasmodium. Probably based on initial observation (cyanotic tongue), Spreull (1905) named it bluetongue and described the disease as incurable and characterised by fever and lesions of mouth and feet. Theiler (1906) proved, by passing infective fluids through a Berkefeld filter, that the aetiologic agent of the disease was probably a virus. The virus was isolated from the blood collected from affected sheep between 1901-1902 in Cape Colony, South Africa. Subsequently, efforts were made to characterise the virus in terms of virulence. Virulence of different strains of BTV isolated from sheep showed great difference. Based on this observation, Theiler (1908) selected a mild strain of BTV and produced a vaccine by serially passaging the virus in susceptible sheep. Theiler's monovalent sheep attenuated vaccine was used in South Africa for 40 years although sometimes it caused harmful effects in vaccinated animals. Apparent failure of monovalent vaccine, led Neitz (1948) to demonstrate that there were multiple types of the virus circulating in nature. He demonstrated in

animal experiments that the vaccine failure was not due to poor immune response against the monovalent vaccine but because of plurality of BTV types circulating in the animals.

The initial observations that the disease was non-contagious and its incidence was more in high insect activity season led to the speculation that it was transmitted by insect vector. Initially a species of *Aedes* mosquitoes was incriminated as harbouring the disease infection (Nieschulz *et al*. 1934). However, Du Toit (1944) demonstrated that infected *Culicoides* midges could transmit the disease to susceptible sheep. Foster *et al.* (1963) proved beyond doubt that *Culicoides variipennis* transmitted BT disease biologically in the United States of America. Several *Culicoides* species were identified to be the vector of BTV in different regions of the world (Mellor 1990).

Bluetongue virus has the distinction of being the first insect-transmitted virus to be isolated in the laboratory. After about a half century of recognition of BT disease in sheep in South Africa, in 1943 the first confirmed outbreak of BT outside Africa occurred in Cyprus in 1949. However, it is believed that periodic unconfirmed outbreaks had occurred in Cyprus as early as 1924 (Polydorou 1985). Perhaps because of exceptional severity, 1943 outbreak served to focus attention on BT and also facilitated recognition in other regions of the world. Subsequently, BT was recognised in Israel in 1951 (Komarov and Goldsmit 1951), in Pakistan in 1959 (Sarwar 1962) and 1963 in India (Sapre 1964).

In the United States of America, the disease was first reported under the name of Sore Muzzle (Hardy and Price 1952). BT apparently first appeared in Texas in 1948 and subsequently in California in 1952 where it was diagnosed as BT with isolation of the causal agent (McKercher *et al*. 1953). A major epizootic of the severe disease occurred due to BTV serotype 10 during 1956-57 in Portugal and Spain in which 179,000 sheep died resulting in 75% mortality rate (Manso-Ribeiro *et al*. 1957). The presence of BTV in Australia in October 1977 (St. George *et al*. 1978) was accepted with skepticism. However, subsequent isolation of multiple serotypes from cattle and insect vector proved beyond doubt that BTV was present in Australia. Similarly, evidence of BTV infection was reported from Malaysia in 1977, in 1979 from China and subsequently, other countries of the region also reported prevalence of BTV infection in ruminant livestock.

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3

The Virus

Bluetongue virus belongs to the family Reoviridae. In 1969, Verwoerd reported that BTV possessed a segmented dsRNA genome. Borden and his associates proposed the name *Orbivirus* for BTV-like group in 1971. *Orbi* is derived from Latin word *orbis* which means ring or circle. The viruses included under BTV-group share some similarities with reoviruses such as dsRNA genome, lack of lipid envelope, their acid lability and genome organisation; however transmission by insect vectors justified their classification under a separate genus within Reoviridae. In 1976 the International Committee on Taxonomy of Viruses officially distinguished genus *Orbivirus* within the family Reoviridae with BTV as type species (Fenner 1976).

Physico-chemical properties

The stability of the virus is different in different physico-chemical environments. Density gradient ultra centrifugation has allowed purification of the virus. The purified virus has been estimated to have about 80% proteins, 20% nucleic acid dsRNA genome (Verwoerd 1969). The base composition has been determined to be 42.4 % G+C value (Verwoerd *et al.* 1979). Removal of one or more proteins from the outer capsid abrogates infectivity of the virus. Monovalent and divalent cations alter outer capsid protein layer at ionic concentrations in pH dependent manner.

The virus is quite stable at 25°C. The virus-positive blood can be stored in OPG solution (potassium oxalate, 5 g; phenol, 5 g; glycerine, 500 ml; and distilled water, 500 ml) at 4°C for a year without any substantial loss of infectivity. Neitz (1948) suggested that the virus should not be frozen slowly at –10° or –20°C because freezing at these temperatures would result in loss of infectivity. Svehag (1963) reported that storage of the virus for one month at 5°C and 25° C resulted in 50% loss of infectivity, while at –70°C there was no demonstrable loss of infectivity. Even when stored at –70°C for 1 and 3 years, 50 % and 5% of infectivity, respectively, was retained (Svehag 1963). Foster and Luedke (1968) showed that undiluted embryo supernatant fluid was very stable at refrigerator (4°C) temperature over a year. Foster and Luedke (1968) also reported that partially purified infected chicken embryo fluids lost infectivity rapidly when stored at 4°C.

Luedke (1969) reported that storage of blood in OPG solution at 4°C for 4 months was necessary to release the virus for titration via yolk sac

route, and addition of lipase in blood and subsequent sonication helped in release of the virus. Howell *et al.* (1967) reported that tissue-culture adapted virus was quite unstable unless suitable biologic stabiliser was added in the medium. They suggested that 50% buffered lactose-peptone solution was most efficient stabiliser. However, it was toxic to the cell cultures. They further reported that infectivity of stock virus strains in buffered lactose-peptone, freeze-dried and stored at –20°C, was negligible after 8 years.

Svehag (1963) reported that thermal inactivation of the virus at 46°C to 56°C, was probably due to protein denaturation, however, he suggested that loss in infectivity at 37° C to 46°C was probably due to inactivation of RNA genome.

Bluetongue virus is resistant to ether, chloroform and sodium deoxycholate (Studdert 1965, Svehag 1966). Chloroform and Genesolv-D are able to partially purify BTV from blood of infected sheep and these solvents have no adverse effect on the virus. Relatively low concentration of trypsin inactivate the virus (Svehag 1966). They reported that the virus was stable at pH 6-8. The virus is inactivated below pH 6 and above pH 8 within a minute. Owen (1964) reported that marked loss of infectivity could be expected at pH 6 to 6.3 and that the survival of BTV in carcass meat appeared to be dependent on postmortem pH change. He further found that the virus persisted for 30 days at 4° C in an ovine carcass where the pH failed to drop below 6.3. No virus was isolated from the ovine carcass in which pH dropped below 5.4. Bluetongue virus was isolated from the bovine carcass 10 days after experimental infection.

Gamma rays irradiation of 1-2 M rad completely inactivates the virus, while irradiation doses above 4 M rad adversely affect the antigenicity of the virus. Bluetongue virus is also inactivated by ultraviolet radiation with in few seconds to one minute. Heating at 56°C also inactivated it.

Virus morphology

Morphologically three distinct particles, viz. virion, core and sub-core particles have been identified. Different particles can be obtained by step-wise removal of major structural proteins. A sedimentation constant of 550 S (Svedburg) and a buoyant density of 1.36 g/cm^2 in CsCl (cesium chloride) has been reported (Martin and Zweerink 1972, Van Dijk and Huismans 1982). Owing to tendency of aggregation, the core like particles are difficult to purify on sucrose gradient. However, in appropriate conditions they have sedimentation constant of 470S. They are stable in CsCl gradients and have a density of 1.40 g/cm^2 (Van Dijk and Huismans 1982). The main feature of core particles is presence of 32 distinct morphological units called capsomers.

Recently 3-D structure of the virus has been studied using cryoelectron microscopy and computer image reconstruction system (Grimes *et al.* 1995, Hewat *et al.* 1992, Prasad *et al.* 1992). The complete virion has 86 nm diameter while core particle has been determined to 69 nm diameter. 3-D reconstruction of complete virion has suggested that it has icosahedral

configuration (Hewat *et al*. 1992) which is in contrast to the previous understanding by negative staining method. The negative staining method indicated fuzzy appearance to outer capsid of the virion (Verwoerd *et al*. 1972). The transmission electron microscopy was also performed by the authors to detect BTV serotype I in BHK-21 cell culture (Fig. 1A, B).

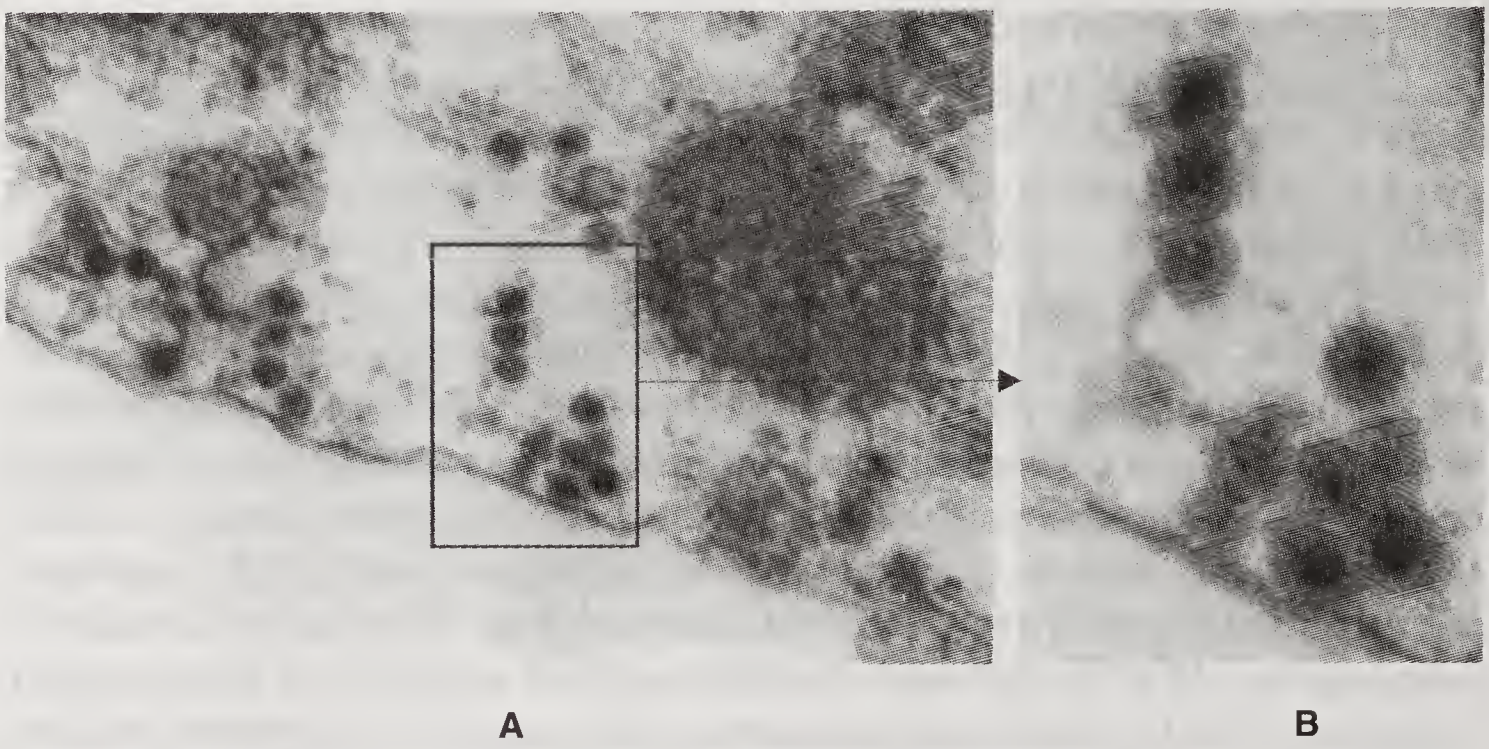

Fig. 1. A: Transmission electron micrograph of bluetongue virus (46, 500 X), B: Enlarged portion of marked area of Fig. 1 A showing double shelled virus particles.

The cryoelectron microscopy has clearly demonstrated that the core particle has icosahedral symmetry with a 13 triangulation number in left handed configuration. The outer layer of the core is composed of clusters of VP7 trimers. In a core particle 780 VP7 molecules has been determined. VP3 forms the inner layer of the core, which has been determined to be featureless. Radius of the central portion of the core is less than 21 nm which accommodates VP1, VP4 and VP6 proteins and genomic RNA segments (Roy 1996). Owing to segmented nature of the genome, each segment can be separated by polyacrylamide gel electrophoresis (PAGE). Electrophoretic migration pattern of genome segments of different isolates may provide some idea about the genomic diversity of the viruses. Whole genome segments are divided into three groups, viz. large (L1-L3), medium (M4-M6) and small (S7-S10). Their migration may vary according to the electrophoretic conditions used. The electrophoretic migration of BTV genome segments may also vary with other orbiviral genomes. Migration pattern of different isolates of the same serotype of BTV may vary. In addition, there may be extra genome segments which can be demonstrated by RNA-PAGE (Fig. 2; Prasad *et al*. 1998).

Viral genome

BTV genome is segmented and composed of 10 discrete ds RNA segments ranging from 0.5 to 2.7×10^6 Daltons. In addition, single stranded

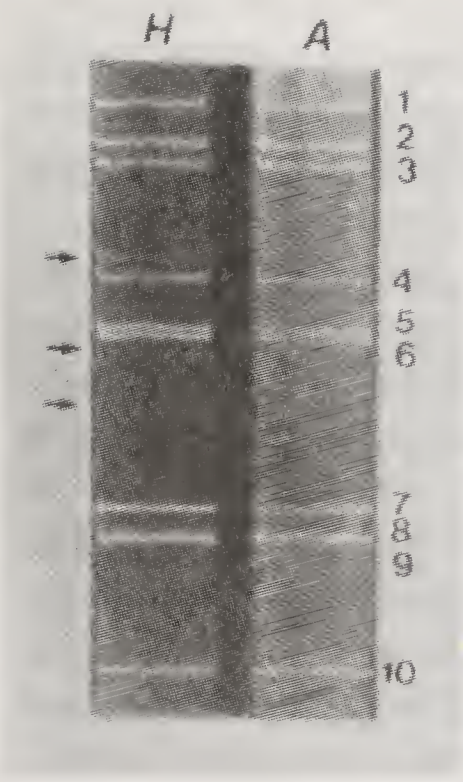

Fig. 2. Electrophoretic migration pattern of two isolates of BTV serotype 1. H: BTV serotype 1 (Hisar isolate) exhibiting 3 extra genome segments indicated by arrows. A: BTV serotype 1 (Avikanagar isolate) showing typical 10 segment pattern of BTV genome.

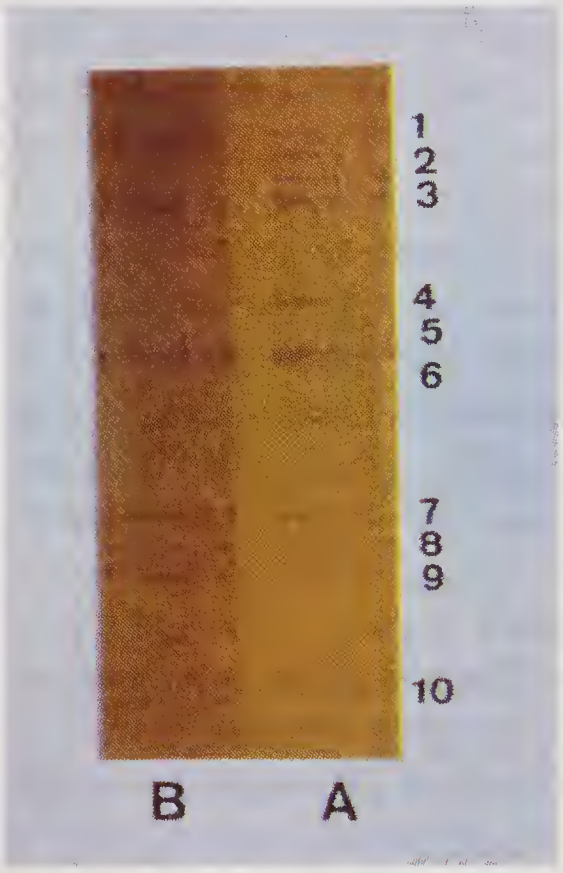

Fig. 3. Migration pattern of genome segments of BTV serotype 18 (Bangalore) and 1. Lane B represents serotype 18 and A as serotype 1. Note the variation in the migration pattern of segment 2, 3, 5, 6, 7, 8 and 9.

mRNA has also been demonstrated in the BTV virion. Each mRNA species is exact replica of positive strand of one of the double strand segment (Huismans and Verwoerd 1973). All the 10 segments of the virus have been cloned, expressed and sequenced. The molecular studies have suggested some common features, including short 5' and 3' non-coding region. The non-coding region varies from 8 to 34 bp (Roy 1989). A sequence of 6 nucleotides at the 3' and 5' ends of all BTV ds RNA segments is conserved (Kiuchi *et al.* 1983, Rao *et al.* 1983, Mertens and Sanger 1985).

Significant variations have been reported in electrophoretic migration pattern of genome segments of different serotypes/ isolates (Fig. 3). However, there is no characteristic migration pattern of any serotype or isolate. Therefore, RNA-PAGE migration pattern can not be used for identification of serotypes (Gorman and Taylor 1985). Variation in electrophoretic migration in PAGE is not accurate reflection of the size of the segment (Pedley *et al.* 1988), and the variation in the concentration of polyacrylamide can affect the migration of genome segments (Mertens and Sangar 1985). These variations may be ascribed to base composition or to secondary structures. On agarose, separation is purely on the basis of size and therefore, no differences in the ds RNA segment migration profile of different BTV serotypes have been reported (Squire *et al.* 1983, Kowalik and Li 1987).

Genome-segments-protein coding assignment

The virus genome has 10 discrete ds RNA segments. Each segment encodes a protein. However, genome segment 10 encodes two related non-structural proteins namely NS3 and NS3A (Mertens *et al.* 1984, French *et al.* 1989). The virus genome codes for two groups of proteins, viz. structural and non structural proteins.

Structural proteins

Structural proteins are the constituents of the virion and have been designated as VP1, VP2, VP3, VP4, VP5, VP6 and VP7. The structure and function of each of these proteins is described briefly based on their location in the virion.

i. Outer capsid proteins: The core of the virus is surrounded by an outer shell composed of VP2 and VP5. The outer shell has a fuzzy shape. However, recent study suggested that it may also have icosahedral symmetry. Both the proteins of the outer capsid have been demonstrated to be associated with the virus neutralising immune response.

VP2: It is encoded by L2 segment of the virus genome and has 111 kDa Mr (Roy 1996). VP2 is nonglycosylated and exhibits haemagglutinating property. The *in vitro* and *in vivo* studies demonstrated that it is involved in the virus attachment with the mammalian host cell receptors (Eaton and Crameri 1989). The studies have also suggested that stripping off this protein from the outer capsid abrogates the infectivity of the virus to the mammalian host cells. This suggested that VP2 is critical for the infectivity. However, it has also been shown that the virus particle devoid of VP2 still remains infective to invertebrate (insect) cells (Cowley and Gorman 1987, Mertens *et al*. 1989). The virus neutralisation studies both *in vitro* and *in vivo* have proved that VP2 is the main protein, which elicits neutralising antibody response in serotype specific manner. The neutralising immune response generated by VP2 is protective against homologous challenge. The VP2 proteins of some serotypes share neutralising epitopes and some degree of cross-protection has been observed amongst a few serotypes. The neutralising epitopes located on VP2 proteins of the serotypes studied so far suggested that these are largely conformational, hence lost during separation and purification of the protein consequently losing the ability to provoke neutralising immune response. The nucleic acid sequence analyses of corresponding gene (segment L2) of different serotypes of BTV have revealed vast differences between serotypes. This is the reason why VP2 is serotype determining protein. The phylogenetic studies have indicated that some serotypes are more closely related while others are distantly related. Both highly variable as well as relatively conserved regions have been identified in VP2 gene of different serotypes. The conserved regions are generally located in carboxyl terminus and middle region. This suggested that this region may be functionally important. The six conserved cysteine, similar hydrophobic profiles and the indicated secondary structures such as alpha helices, b- strands and turns suggested common phylogenetic origin of BTV serotypes (Roy *et al*. 1990a).

The cloning and sequencing of Indian isolates of BTV serotype 1 in the author's laboratory indicated 99 % sequence homology among BTV-1 Avikanagar, Sirsa 3 and Chennai isolates. BTV-1 Sirsa1 exhibited 11-13% divergence from BTV-1 Avikanagar, Sirsa 3 (S3) and Chennai (M) isolates. Six base additions and 1 base deletion was observed in BTV-1 Sirsa1 isolate. The VP2 gene sequences (1240-1844 bp region) of Indian isolates of BTV-1

have high sequence homology with BTV-1 Australian isolates than with BTV-1 South African isolates and other BTV serotypes (Dahiya *et al* 2004). *In silico* restriction pattern of Indian isolates of BTV-1 was same as that of *in vitro* restriction profile of VP2 gene (604 bp RT-PCR product) using *Taq*I RE. RE profile analyses of variable and conserved regions of VP2 gene with *TaqI* and *XmnI* showed that the Indian isolates of BTV-1 are more closely related to BTV-1 Australian isolates than to BTV-1 South African isolates (Dahiya *et al* 2005, Fig. 4). Phylogenetically BTV-1 Indian isolates form one group closely related with BTV-1AUS isolates followed by BTV-1SA isolate. Dash (2000) cloned and sequenced different regions of VP2 gene of Indian isolate of BTV serotype 23 and suggested that there was around 90 % sequence similarity among different isolates of BTV serotype 23. Tembhurne (2004) also sequenced VP2 gene of BTV 18 and BTV 23 isolated from India and compared with other isolates of the same serotypes. There were vast differences between serotypes while there was not much variation with serotypes.

VP5: It has a molecular mass of 59 kDa and has 526 amino acids. The information is scanty on this protein. It is less variable as compared to VP2. Amino acid sequence analyses have suggested that it has high alanine and leucine and a very little tryptophan. Some reports have indicated that this protein may be glycosylated (Yang and Li 1993). Though it is located on the outer capsid, it does not appear to have ability to bind with

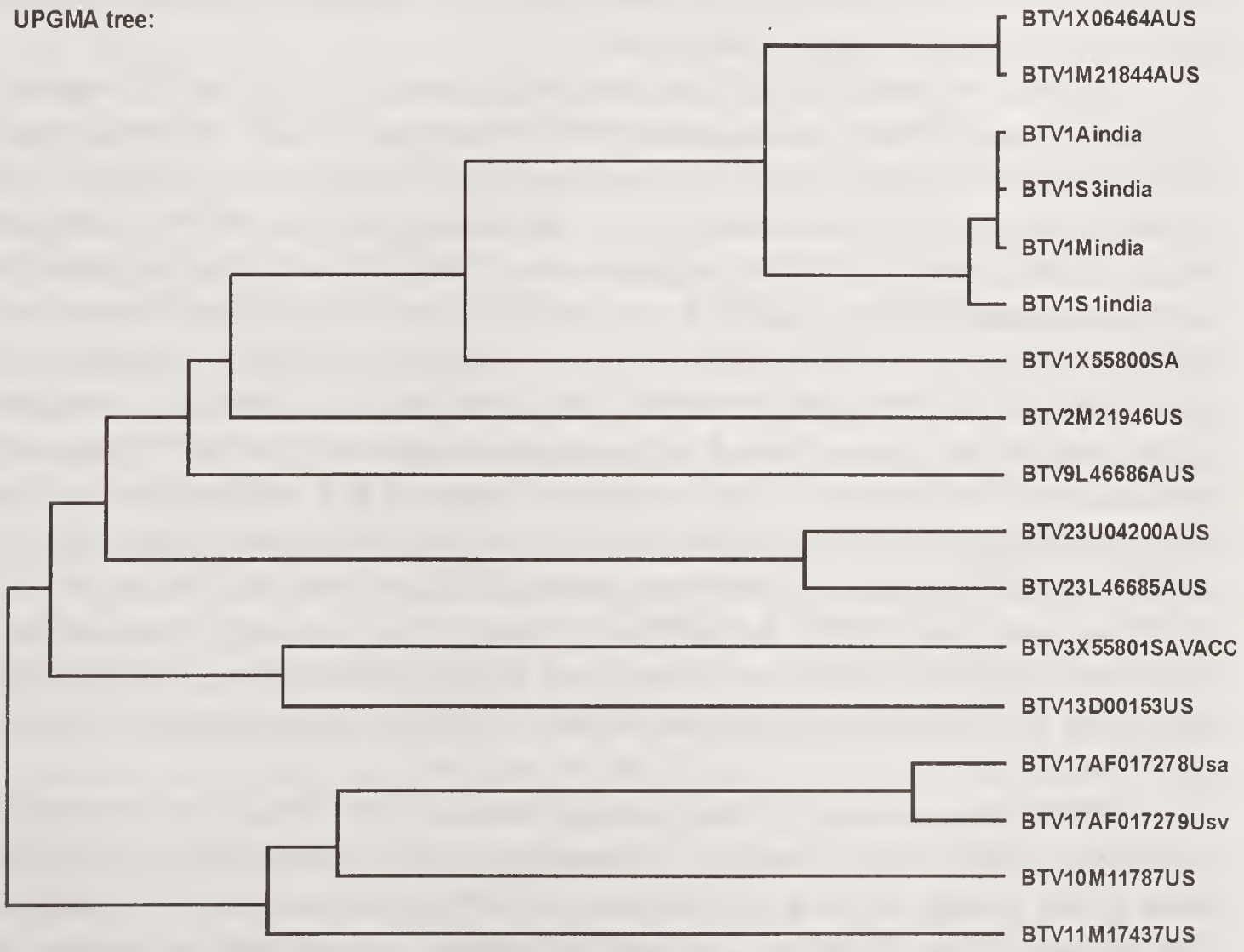

Fig. 4. Phylogenetic relationship amongst different BTV serotypes based on 1240-1844 bp (604 nucleotides) of VP2 gene.

neutralising antibodies directly or induce neutralising antibodies. Antibodies raised against VP5 does not neutralise the virus. However, in contrast to this the combination of VP5 with VP2 enhances neutralising immune response in vaccinated animals (Roy *et al*. 1990b). This indicated that perhaps VP5 indirectly enhances neutralising antibody response by interacting with VP2 and affecting its conformation consequently influencing its immunogenicity. Possibly it has a role in eliciting cellular immune response. Forzan *et al.* (2004) have suggested that protein VP5 acts as a membrane permeabilization protein that mediates release of viral particles from endosomal compartments into the cytoplasm. They further demonstrated that VP5 can also act as fusion protein and induce syncytium formation if fused to a membrane anchor and expressed on the cell surface. The fusion activity is dependent on pH and is triggered by short exposure to low pH (Forzan *et al* 2004). Tembhurne (2004) cloned and sequenced VP5 gene of BTV serotype 18 and 23 isolated from India and studied phylogenetic relationship with BTV isolates from other parts of the world.

Inner capsid (Core) proteins: Inner capsid is composed of 5 proteins, viz. VP1, VP3, VP4, VP6 and VP7. The minor core proteins are VP1, VP4 and VP6, and are present in less abundant number in the virion, while VP3 and VP7 are the major core proteins and are present in high copy number. VP1, VP4 and VP6 are located in the inner most part of the core while VP3 and VP7 form outer surface of the core. VP1, VP4 and VP6 are highly conserved across the BTV serotypes. Characteristics and functions of each one of these are described here.

Minor core proteins: VP1: It is the largest protein coded by L1 segment of the genome. VP1 is minor core protein having 150 kDa molecular mass and an important component of the virion. VP1 is highly basic protein with positively charged carboxy terminus. It has been predicted to be an enzyme with RNA dependent RNA polymerase activity that enables the virus to synthesise mRNA from ds RNA template. It is rich in hydrophilic residues such as serine and threonine and the aromatic residues tyrosine and phenylalanine as compared to other viral proteins. It is present in very low copy number in virion. Based on its location, low copy number, size and presence of GDD motif (characteristic of other RNA polymerases) it has been considered as RNA polymerase. The *in vitro* studies using baculovirus expressed VP1 have very clearly suggested RNA polymerase like activity in it (Urakawa *et al.* 1989). In addition it has some homology with B-chain subunit of *E. coli,* tobacco chloroplast RNA polymerase, *Saccharomyces cerevisiae* RNA polymerase II and III, and *Drosophila* polymerase II (Roy *et al*. 1988).

VP4: It is a minor 761 kDa protein of the inner core coded by segment 4 (M4) of the genome. The amino acid sequence analysis indicated that it has a high content of histidine and tryptophan, and low content of glutamine. It has a strong hydrophilic domain at carboxyl terminus as potential leucine zipper is present near this end (Hwang *et al*. 1993). Mertens *et al.* (1992) have reported that VP4 catalyses capping of BTV

mRNA and suggested that guanylyl transferase activity of BTV is associated with VP4. This observation has been supported by studies conducted using recombinant VP4 (Le Blois *et al.* 1992).

VP6: It is a nucleic acid binding protein of 35 kDa coded by S9 segment of the viral genome. It has two domains separated by a glycine rich region. Bluetongue virus derived conventional or recombinant baculovirus expressed VP6 has strong affinity to ss RNA and ds RNA. The amino acid sequence analysis has shown that it has motif common to helicases. This activity may be involved in unwinding of ds RNA genome before synthesis of mRNA species. VP6 protein has a number of activities, including nucleoside triphosphatase, RNA binding and helicase activity (Stauber *et al.* 1997, Kar and Roy 2003).

Major core proteins: VP3: It is a 103 kDa protein with 901 amino acids. This protein is encoded by L3 segment of the genome. It is responsible for formation of inner scaffold for the laying of surface layer of VP7 on the core. VP3 also interacts with other 3 minor proteins of the core during assembly and incorporation of these into core of the virus. It is highly conserved across the BTV serotypes and other orbiviruses such as EHDV, AHSV-4. The studies on recombinant baculovirus expressed VP3 have suggested that it has group specific antigenic determinants (Inumaru and Roy 1987) and reacts with all anti-BTV sera while weak reaction has also been reported with EHDV and AHSV (Le Blois *et al.* 1991). Tamilselvan (2004) amplified 648 bp sequence representing 1026-1673 nt of VP3 gene of 6 Indian isolate of BTV and sequenced it for determination of topotypes of the BTV circulating India. The phylogenetic analysis of the sequence data suggested that Indian isolates of BTV belonged to Australasian topotype.

VP7: It is a group specific protein of 38 kDa M_r. VP7 is coded by segment 7 (S7) of the genome. It is a major structural protein located on the outer surface of the core and comprised about 36 % of the total core proteins (Huismans *et al.* 1987). It has major group specific antigenic determinants and highly conserved across the BTV serotypes. It is closely related to EHDV and AHSV virus. The amino acid sequence analysis has revealed that it is deficient in charged amino acids (AA) and highly hydrophobic. The AA sequence analysis of VP7 has further suggested that there is only one lysine, which is unique since other BTV proteins are rich in lysine. VP7 is rich in alanine, methionine and proline. VP7 is predominantly located on the surface of the core. However, Hyatt and Eaton (1988) demonstrated that VP7 may be accessible from the surface of the virion. Large amounts of VP7 have been produced using both eukaryotic as well as prokaryotic expression systems. Since sera against all the serotypes of BTV react with VP7 produced conventionally or recombinant, its use as diagnostic antigen has been recommended (Naresh *et al.* 1996). Anti-serum against VP7 also reacts with EHDV and AHSV, though weakly. This protein has been purified and crystallised (Grimes *et al.* 1995). VP7 attaches with insect cell receptors of the virus. Possibly this is the reason why core particles are infectious to insect cells while non-infectious to mammalian cells.

Tiwari *et al.* (2000) characterized Indian isolates using PCR and restriction endonuclease analysis of VP7 gene. Ramesha (2003) cloned and sequenced the coding sequence of VP7 gene of three Indian isolates of BTV (BTV-1A, BTV-1H and BTV-18B) and reported that Indian isolates have high sequence homology with South African and Austrian isolate than American, Chinese and French isolates. Among Indian isolates belonging to three different serotypes, there is 3-8 % sequence divergence in VP7 gene sequence and there is 5 % sequence divergence between the two isolates of serotype 1, which were isolated from different geographical areas at different times. Hence they form single topotype. Phylogenetically serotype 1 isolates formed a separate monophylatic group whereas serotypes 18 and 23 formed separate monophylatic group with Chinese isolates, South African and Australian isolates (Fig. 5). An *in silco* restriction analysis of VP7 gene sequence of these Indian isolates is quite similar to that of South African and Australian isolates (Ramesha 2003).

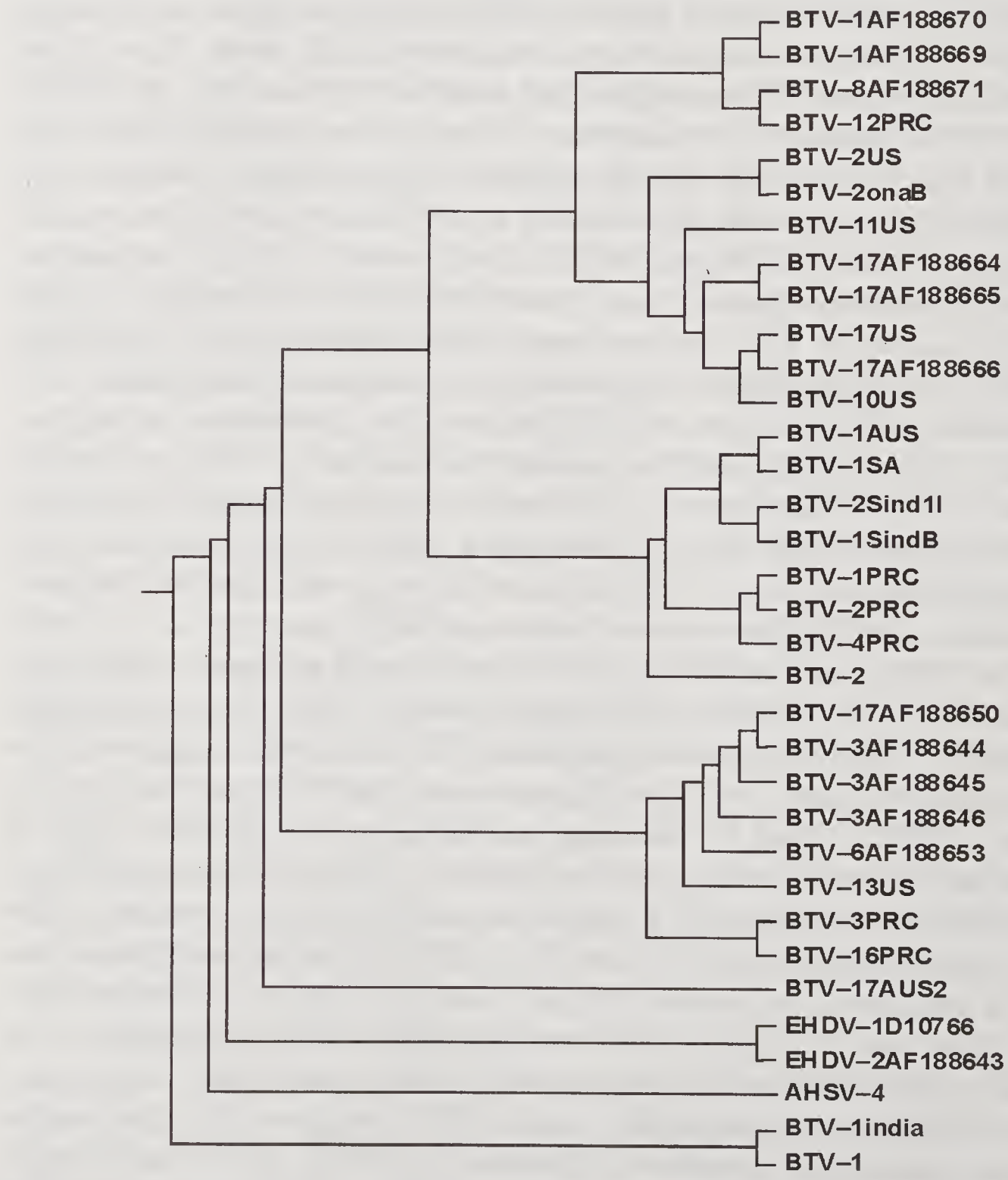

Fig. 5. Phylogenetic relationship amongst different BTV serotypes based on nucleotide sequence of VP7

Non-structural proteins

BTV encodes at least 4 non-structural proteins. Of the 4 non-structural proteins which have been demonstrated in BTV infected vertebrate as well as non-vertebrate cells, NS1 and NS2 are the major proteins and are synthesized in abundance in infected cells. The 2 other non-structural proteins NS3 and NS3A are closely related and are produced in small amounts in the infected cells (French *et al*. 1989, Huismans 1979, Eaton *et al*. 1991, Huismans and Els 1979). The nucleic acid sequence analysis has suggested that there is 96% homology between NS proteins of different serotypes of BTV. The synthesis of NS1 and NS2 in infected cells is evidenced by tubules and granular virally induced inclusion bodies (Roy and Gorman 1990). The presence of these virus specific structures has been demonstrated throughout the cytoplasm of the infected cells. However, their presence has been shown more abundantly near the nucleus. The brief description of individual non-structural proteins and their possible role in the life cycle of the virus has been described here.

NS1: It is coded by genome segment 6 (S6) and has a M_r of 64 kDa. Lee and Roy (1987) provided complete nucleotide sequence of segment 6 (NS1 gene) of BTV serotype 10. Subsequently, Gould *et al.* (1988) provided nucleotide and deduced sequence of NS1 of Australian and South African bluetongue serotype 1. NS1 is synthesised in large amount as compared to other virally coded non-structural proteins. Since it is highly conserved across the different serotypes of BTV, it is potential group specific diagnostic antigen. It has low content of charged AA and rich in cysteine (Roy 1996a). Clearly defined several hydrophobic regions have been identified in the protein (Nel *et al*. 1990). When NS1 gene was expressed in baculovirus, it formed tubular structures in *Spedophora frusiperda* cells confirming that tubules are multimeric structures of NS1 (Urakawa and Roy 1988). Cryoelectronmicroscopic studies have revealed that tubules are on an average 52.3 nm in diameter and up to 1,000 nm long with a helical configuration (Hewat *et al*. 1992). NS1 forms a dimeric structure and tubules are composed of these structures. The deletion and site directed mutagenic studies have suggested that both C and N termini cysteine at amino acid 337 and 340 are essential for tubule formation. Tubular structures are formed due to polymerization of a single 64 kDa non-structural protein, NS1. Owens *et al* (2004) have recently suggested that NS1 may be involved in the translocation of newly synthesized viral particles to the plasma membrane. They have further suggested that NS1 is also involved in cellular pathogenesis and morphogenesis of the virus (Owens *et al* 2004).

NS2: It is coded by S 8 genome segment and has M_r of 41 kDa. NS2 is the only phosphorylated protein coded by BTV genome (Devaney *et al*. 1988, Huismans 1979). It is rich in charged AA. The protein is generally hydrophobic. The inclusion bodies observed in BTV infected cells have been demonstrated to be the aggregates of NS2 protein molecules (Hyatt and Eaton 1988, Thomas *et al* 1990). The expression of NS2 gene in baculovirus has confirmed that inclusion bodies are composed of NS2 proteins (Thomas

et al 1990). The *in vitro* studies have indicated that NS2 protein is phosphorylated at specific serine residues particularly at AA 185 and 308. This protein has ability to bind with ssRNA. However, there is no evidence of its binding with ds RNA (Zhao *et al*. 1994). Role of this protein in the life cycle of the virus remains unexplored.

NS3: This is coded by genome segment 10 (S10) and has M_r of 25 kDa. NS3 has potential for glycosylation at AA 63 and 150. Immunohistochemical investigations have indicated that it is transported to Golgi apparatus and then to cell plasma membrane in presence or in absence of tunica mycin. This observation suggested that carbohydrate is present. The transient expression studies have also confirmed that NS3 and NS3A proteins produced in vertebrate and non-vertebrate host cells are glycosylated and can be converted into heterologous polylactosaminoglycon proteins (Wu *et al*. 1992). The NS3 protein expressed by recombinant vaccinia virus has been demonstrated to be associated with intracellular, smooth surfaced vesicles as well as plasma membrane (Hyatt *et al*. 1991). Hyatt *et al.* (1992) have further reported that when virus like particles are assembled in presence of NS3 protein, the particles are secreted by budding through the cellular membrane. These studies suggest possible role of NS3 in final stages of viral morphogenesis and the release of the virions from the infected cells. Beaton *et al.* (2002) have suggested that NS3 may be involved in virus egress pathway. They demonstrated that NS3 interacts with P11(Calpactin light chain), part of the annexin II complex that is involved in exocytosis. C terminal domain of NS3 interacts with VP2 of the fully assembled virus particle, suggesting that NS3 forms a bridging molecule that draws assembled virus into contact with cellular export machinery (Beaton *et al.* 2002).

NS3A: It is a 24 kDa protein coded by genome segment 10, and has 216 AA. The molecular studies have provided strong evidence that NS3 and NS3A are closely related and are products of the same gene. Both the proteins are synthesised by alternative translation initiation sites (French *et al*. 1989). Hyatt *et al* (1993) reported that release of bluetongue virus like particles from the insect cells is mediated by NS3/NS3A protein.

Replication of the virus

Bluetongue virus replicates in a variety of vertebrate and non vertebrate cell types. It replicates in the cytoplasm of the cells. With the availability of transmission electron microscopy and X ray defraction techniques, it has become possible to study the virus replication cycle in greater detail. Right from the attachment of the virus with specific receptor on surface of susceptible host cell to the release of newly synthesised virions, several steps are required to complete the replication cycle of the virus.

1. Adsorption: The viral isolates from ruminants and insect vectors replicate poorly in mammalian as well as insect cell cultures. However, field isolates can be adapted to grow in high titres by several serial passages in the cell cultures. The host cell receptor which allows binding of the virus

has not been fully characterised. However, some insight has been gained by using the virus-erythrocytes interaction as model system. BTV binds with specific sialic acid containing serine linked oligosaccharides in the glycophorins of human and a variety of animal erythrocytes (Eaton and Crameri 1989). The binding of the virus to the vertebrate host cells including erythrocytes is mediated by VP2 protein, which is located on the outer surface of the capsid. The incomplete virus particles attach very poorly to vertebrate cells (Huismans *et al.* 1983). It is now very well known that VP2 is the main binding protein to the host cells. Contrary to the mammalian host cells, invertebrate cells are easily infected by core particles. This suggests that VP2 is not required for infection to invertebrate cells. Since VP7 is a major core protein, it appears to be the viral protein responsible for attachment with the invertebrate cells. VP7 is a RGD motif similar to some picorna viruses.

2. *Endocytosis:* Once the virus attaches to the cytoplasmic membrane of the host cells, the process of internalisation starts. The virus may enter the cells either by direct penetration of cytoplasmic membrane or by receptor mediated endocytosis followed by penetration either by endosomal or lysosomal membrane. The presence of the virus shortly after infection has been observed by several workers (Lecatsas 1968, Cromack *et al.* 1971). The process of entry of the virus has been extensively studied using electron microscopic and immuno-histochemical methods. The virus binds receptor characterised by clathrin. The clathrin coated cytoplasmic membrane surface grows as a coated pit which finally invaginates and detaches from the cell surface yielding a coated vesicle and rapidly loses the clathrin coat. Then the vesicle may fuse to form large electron lucent vesicle or endosome (Eaton *et al.* 1990). Their studies clearly suggested that the virus enters cytoplasm by penetration of endosomal membrane, and BTV is converted into core within one hour of infection. This observation is in accordance with morphogenesis findings that the uncoating of the virus occurs in endosomes. The removal of both outer and inner capsid layers is essential for activation of virion transcriptase (Van Dijk and Huismans 1980). Acidic pH around 5 is required for uncoating of the virus. The virus coat proteins appear to be responsible for uncoating of the virus either at cytoplasmic membrane or endosomal membrane. After uncoating, viral coded macromolecular synthesis starts that eventually leads to synthesis of new virions.

3. *Multiplication of the virus:* After release of the viral genome segments in the cytoplasm, transcription starts followed by protein synthesis in the ribosomal complex. The viral inclusion bodies have been demonstrated in the cytoplasm where synthesis of viral coded components occurs. With the availability of very high resolution electron microscopy, the minute details of the virus replication cycle have been observed. Immunocytochemical and electron microscopy has demonstrated that the virus forms several inclusions in the cytoplasm of the infected cells. Virus tubular structure composed of non-structural proteins and a large number of VP2 aggregates associated with cytoplasmic skeleton.

Release of the virus from infected cells

The mechanism of release of BTV from the infected cells has not been fully understood. There may be more than one way of release of the newly synthesised virions. Death and subsequent lysis of the infected cells appears to be the most predominant way of the release of virions. BTV is released within 4-5 hours post infection of MDBK cells and thousand fold increase in the virus titre at 15 hours post infection as compared to the cell associated virus. This clearly indicated that the virus is released in culture medium without lysis of the cells (Cromack *et al.* 1971). However, in other cell culture system, a large proportion of the virus remains cell associated (Howell and Verwoerd 1971). Some reports the presence of lypoprotein envelope suggesting release of the virus by budding (Bowne and Jochim 1967). Similar observations were made by Prasad *et al.* (1994). Lecatsas (1968) reported a third method of release of virus. The virus particles may be released due to discontinuties in the plasma membrane. Similar observations were made by the authors (Fig. 6).

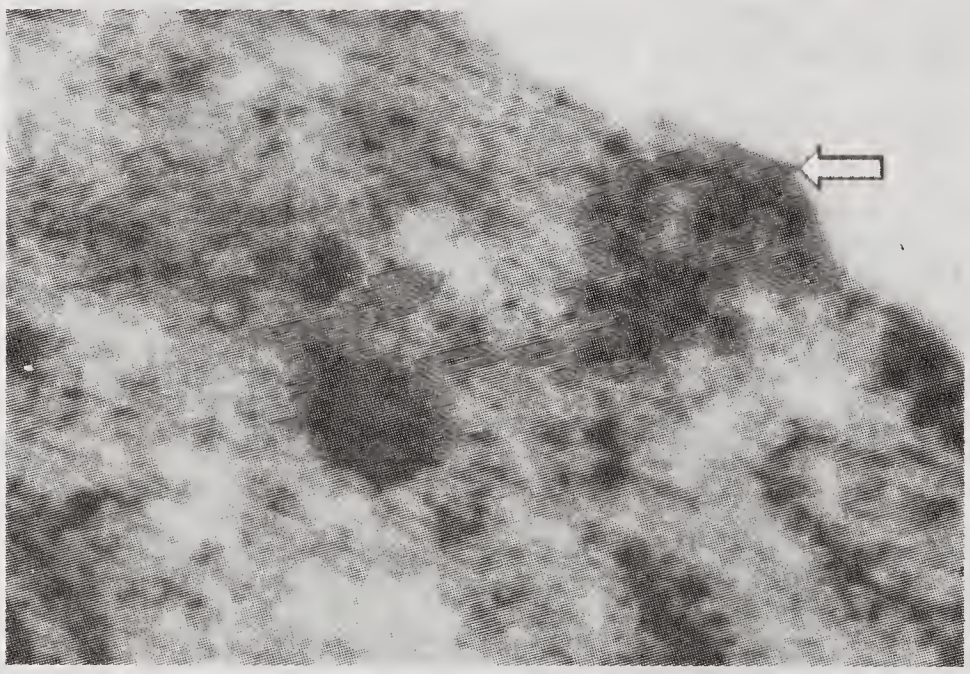

Fig. 6. Transmission electron micrograph exhibiting release by the rupture of cytoplasmic membrane (86, 000 x).

Electron microscopic and immunocytochemical techniques have allowed detailed study of morphogenesis and release of the virus. The virus released by budding may be unstable and they may lose their envelope quickly. The release of BTV by budding was demonstrated by electron microscopy (Fig. 7).

The use of trypan blue exclusion assay has made it possible to study viability of the infected cells at different time intervals. The release of the virus particles was first observed at 6 hours post infection (PI). The maximum release occurred between 12-24 hours post infection (PI). In spite of the release of the virus, the infected cells remained viable as evidenced by trypan blue dye exclusion assay. About 95% cells remained viable in the infected cultures up to 24 hours PI. Similarly integrity of the cells has been studied by ^{51}Cr release assay. This assay also suggested that the infected cells were viable (Hyatt *et al.* 1989). Both these assays strongly indicated that BTV may also be released by other ways than cytolysis and budding.

Virus/and its components associated with intracytoplasmic components

At least three distinct virus-associated structures have been

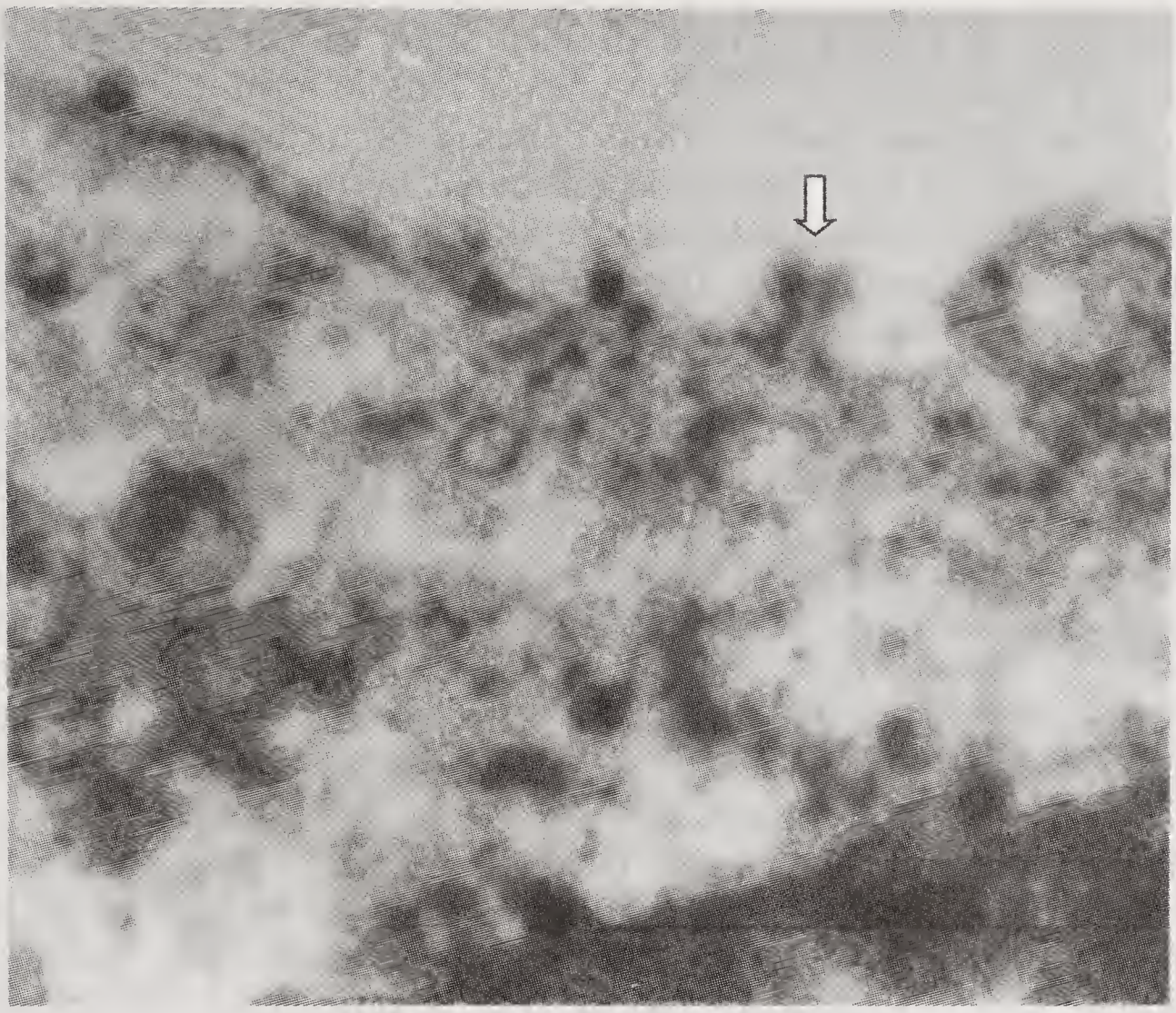

Fig. 7. Transmission electron micrograph showing release of the virus by budding. (72, 000 X)

demonstrated by high resolution electron microscopy and immunocytochemical techniques. These include virus tubules, virus inclusion bodies and virus and virus like particles. Formation of each one of these is briefly described here.

Virus tubules: Electron microscopy has allowed the detailed study of the virus infected cells. Rod shapped structures have been observed in the late stage of the infection in the cytoplasm (Fig. 8). The tubular structures have been purified and idenfied as aggregates of non-structural proteins (Huismans and Els 1979). The immunological studies using monoclonal antibodies have revealed that tubules are predominantly composed of NS1 protein. However, small amounts of VP3 and VP7 can be detected in tubules (Eaton *et al*. 1988, Hyatt and Eaton 1988). The density gradient

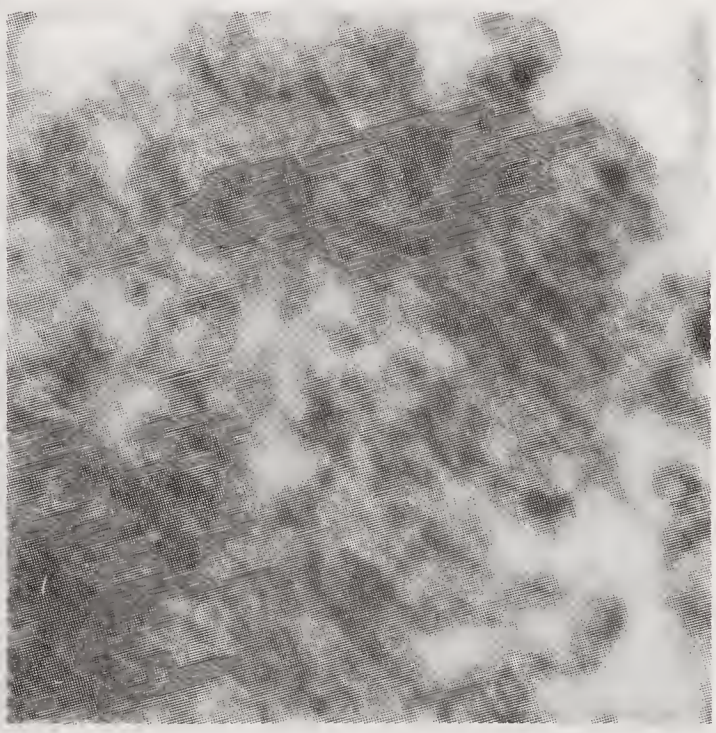

Fig. 8. Transmission electron micrograph of NS1 protein which is seen as rod shaped tubules.

purification of tubules allowed detailed study of the morphology and structural organization of different units of NS1 to form tubular structure.

Virus inclusion bodies (VIBs): Presence of inclusion bodies in the infected cells has been amply demonstrated by high-resolution electron microscopy and immunofluorescence studies. The inclusion bodies are intracytoplasmic and contain dense core like particles. This indicated that probably the virus morphogenesis takes place within VIBs. The presence of nucleic acid within VIBs has been demonstrated by acridine orange staining (Bowne and Jochim 1967). Electron microscopic observation of the section of VIBs has indicated that the particles within VIBs lack outer layer while the particles seen at the periphery are bishelled. The immunogold labelling technique has allowed identification of virus proteins present in the VIBs. The monoclonal antibodies against VP3 and VP7 bound to the structures present in the matrix as well as periphery of the VIBs. However, the presence of VP2 in VIBs has been controversial. Immunogold studies suggested that VP2 is present in the particles in periphery of VIBs (Hyatt and Eaton 1988). This observation strongly indicated that VP2 is probably added on the core particles at the periphery of VIBs (Gould 1988). Monoclonal antibodies against NS2 react at the surface of VIBs in immunogold and immunofluorescence labelling studies. NS2 binding has also been demonstrated in the core of VIBs. NS2 is phosphorylated protein and has tendency to bind RNA suggesting its role in the viral RNA synthesis. Unlike NS2, presence of NS1 has not been demonstrated in matrix of VIBs. The cytoskeleton staining has indicated that NS1 is present in the periphery of VIBs or in the virus particles leaving VIBs. The electron microscopic studies have suggested that tubular structures are present in abandance throughout the cytoplasm of the infected cells as early as 6 hours post infection before appearance of significant VIBs and virus particles. This indicated that NS1 might not be involved in the morphogenesis of the virus.

Virus and virus like particles: Electron microscopy has allowed assessment of different structures including virus and virus like particles (Fig. 9). The particles that react with anti-VP2 antibodies have been identified in several places in the cytoplasm. The infectious virus particles are present in the cytosol and can be released by treatment of cells with NP40. The virus particles associated with cytoskeleton show variations in the morphology.

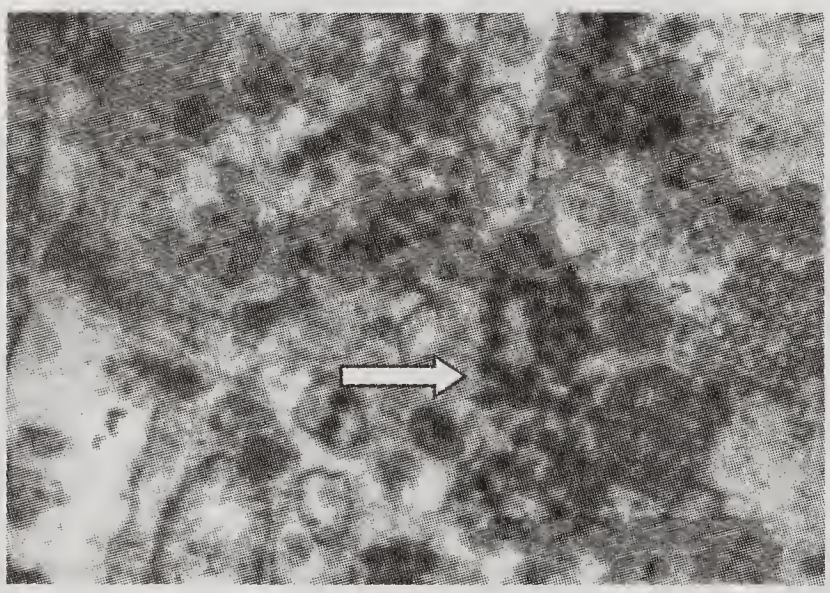

Fig. 9. Transmission electron micrograph showing numerous virus particles in the cytoplasm of infected BHK 21 cells. (62, 000 X).

Bluetongue virus has been investigated as a prototype of Orbiviruses during past 50 years to

understand biology of this group of viruses. As has been described in the chapter, great insight has been gained into molecular organization of different protein molecules in both inner and outer capsids and the organization of the ds RNA segments in the core particles. This information has been crucial in understanding the replication of viral genome and synthesis of structural and non-structural proteins of the virus and mechanism of their assembly into the new virions and their subsequent release from the infected cells. Complete nucleotide sequencing of some serotypes of BTV has been done which has been helpful in understanding the phylogenetic relationship between BTV circulating into different regions of the world. However, the sequence information of several isolates from different parts of the world is still lacking. Therefore, efforts are required into developing a comprehensive nucleotide sequence data bank for all the genes of all the serotypes of viruses isolated from different geographic regions of the world. Availability of such information would be of great help in designing molecular diagnostic tools and development of suitable vaccines.

All members of the genus *Orbivirus* are prone to reassortment in vertebrate as well as invertebrate hosts. During past over 30 years bluetongue virus and its different serotypes have been molecularly and immunologically characterized. The basic molecular mechanism of virus replication, assembly, morphogenesis and subsequent release from the infected host cells has been extensively studied both in vertebrate and nonvertebrate host. However, all the genes of all the serotypes and topotypes of different episystems are yet to be sequenced. If international monitoring, surveillance and reporting system in the form of international network is to be put in place, molecular characterization of topotypes is very important. The availability of the nucleotide sequences would encourage development of more refined molecular tools for monitoring the virus dynamics in different episystems and their transmission between episystems.

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4

Epizootiology

Bluetongue virus has very diverse host range including wild and domestic ruminants and transmitted by several species of *Culicoides* midges. Distribution of BTV infection is dependent on the prevalence of competent vector in the ecosystem. Natural and man-made changes in the environment have great influence on prevalence of BTV in domestic and wild ruminants. Several macro-and micro-environmental factors govern epizootiology of BTV. Global changes have profound implication on prevalence of arboviral diseases. As a consequence of burning of fossil fuel and other habits of industrialized countries, concentration of CO_2, methane and carbon monoxide have gradually increased to unprecedented level during over past 2,000 years. The phenomenal increase in CO_2 and other gases has potential to induce green house effect leading to global warming. In aggregate, these changes are predicted to modify temperature, rainfall, wind pattern and sea level directly. These changes are likely to affect ecology including interaction of vectors, vertebrate host and viruses. The ecology can also be influenced by population growth, livestock management practices, poverty and social habits.

The distribution of BTV is focal and it depends primarily on the range of the reservoir, midges and domestic animals. The range of the midges depends upon temperature, monsoon and water availability. The midge, sheep, cattle and other ruminants will survive global warming. The midge is capable of translocation to a new geographic area as warming occurs and the midge and ruminants are capable of adaptation to a new geographic area. The midges may be restricted to dung breeding while other species that may not be able to withstand the ecological changes may become extinct. One cannot be sure that BTV will necessarily replicate better at warmer temperature. Further one can reason that an increase in ruminants' population is analogous to increased human population. More human population means increased demand for meat that means increase in livestock and therefore, increase in dung meaning more breeding sites for midges coupled with global warming. This may lead to increased prevalence of BTV and shifting of *Culicoides* midges and BTV infection further to temperate regions of the northern and southern hemispheres.

In the past few decades, enormous information has accumulated in the literature regarding the epizootiology. BTV has been reported from almost all the continents except Antarctica. (Fig. 10). Molecular tools have been used to study molecular epizootiology in relation to geotype/topotypes and

competence of the insect vectors. This chapter would briefly describe all the dimensions of epizootiology of BT. For clarity, the information has been provided continent-wise.

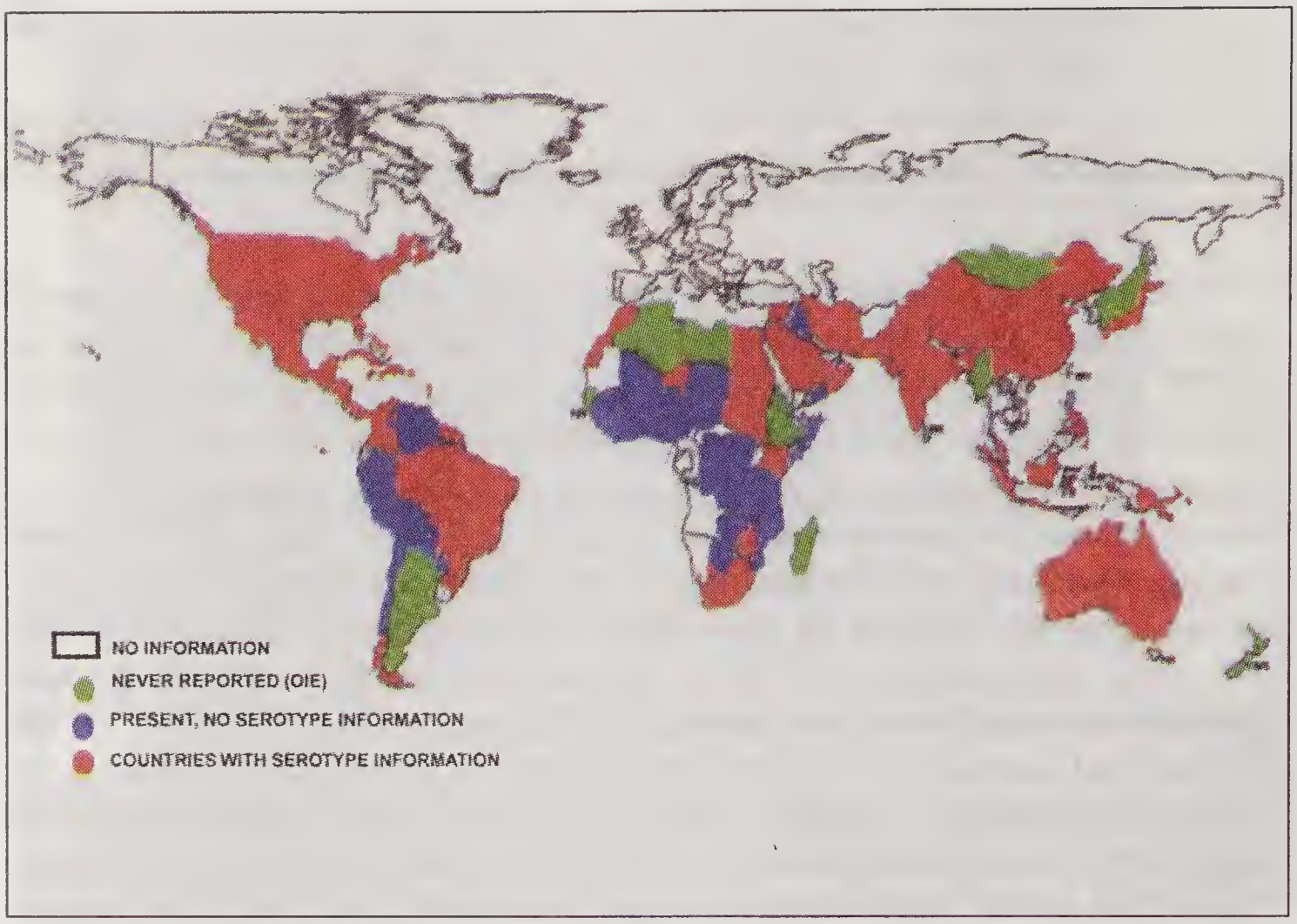

Fig. 10. Global distribution of bluetongue. (Modified from Hawkes, 1996)

Africa

Bluetongue virus is not only endemic but also believed to have originated from Africa. It was first detected in South Africa more than 100 years ago in Merino sheep. Later on it spread to other countries in African continent. The disease was reported from Ghana in 1918 while it was detected for the first time in 1943 in Kaduna state of Nigeria. Subsequently, BTV serotype 6, 10, and 16 were isolated from *Culicoides* sp. while BTV 7 was isolated from *Crocidura* sp. in Nigeria (Lee *et al.* 1974). An extensive study conducted on the sero-prevalence of BTV in Nigerian animal population indicated the presence of BTV antibodies against serotypes 2, 3, 6, 7, 9, 10, 12 and 16. The study also suggested that BTV did not cause frank clinical disease in cattle and goats. However, both of these animal species serve as reservoir of the virus (Tomori *et al.* 1992). The virus infection has been reported from many other countries of African continent (Table 1). The BTV isolates detected in Africa are presented in Table 2.

Asia

BTV infection is widely prevalent in Asian countries including Middle East. Based on WHO-FAO-OIE Animal Health Report (Hassan 1992) the BTV infection is prevalent in livestock population of Israel, Jordon, Syria,

Table 1. Distribution of bluetongue in African continent

Country	Current distribution	Reference
Botswana	Widespread	Mushi *et al.* (1992)
Côte d'Ivoire	Widespread	Formenty *et al.* (1994)
Cameroon	Widespread	Ekue *et al.* (1985)
Egypt	Widespread	Ayoub & Singh (1970)
Ethiopia	Widespread	Lefèvre and Calvez (1986)
Gabon	Widespread	Lefèvre and Calvez (1986)
Gambia	Widespread	Goossens *et al.* (1998)
Guinea	Widespread	Butenko (1996)
Kenya	Widespread	Davies *et al.* (1992)
Lesotho	Present	OIE (1999)
Morocco	Present	Hawkes (1996)
Madagascar	Widespread	Ferreira and Rosinha (1986)
Mali	Widespread	Maiga and Sarr (1992)
Mauritania	Widespread	Lefèvre and Calvez (1986)
Malawi	Widespread	Haresnape *et al.* (1988)
Mozambique	Widespread	Kanhai and Silva (1981)
Namibia	Present	OIE (1999)
Niger	Widespread	Mariner *et al.* (1989)
Nigeria	Widespread	Bida and Eid (1974)
Reunion	Widespread	Barré *et al.* (1985)
Sudan	Widespread	Abu Elzein (1985)
Senegal	Widespread	Lefevre and Taylor (1983)
Somalia	Widespread	Lefevre and Taylor (1983)
Chad	Widespread	Lefèvre and Calvez (1986)
Tunisia	Present	OIE (1999)
Tanzania	Widespread	Hyera and Lyaruu (1995)
South Africa	Widespread	Nevill *et al.* (1992)
Zambia	Widespread	Mweene *et al.* (1996)
Zaire	Widespread	Lefèvre and Calvez (1986)
Zimbabwe	Widespread	Anderson and Rowe (1998)

Oman, Yemen, India, Pakistan, Bangladesh, Myanmar (formerly Burma), Malaysia, Indonesia, Papua New Guinea, China and Japan (Table 3). Serological evidence was recorded in Lebanon during 1989 while BTV serotype 1 and 12 were isolated from clinical cases of livestock from Egypt. Serologically BTV 1, 3, 8, 9, 15, 16, 20 and 23 serotypes are present in Malaysia. Malaysian bovine and ovine are comparatively resistant to BTV infection as compared to exotic breeds. Similarly BTV is widely prevalent in Indonesia affecting buffaloes, cattle, sheep and goats. Livestock at higher altitude have a lower prevalence of infection than low altitude areas. A nation-wide survey of indigenous ruminants of Indonesia showed an overall 49% prevalence of reactors using AGP test varying from 26% prevalence in sheep to 74% in buffaloes. AGP test reactors were further tested by SN test and found serotypes BTV 1, 12, 17, 20 and 21 (Sendow *et al.* 1992). Study by Sendow *et al.* (1993) on sentinel cattle of Indonesia reported serotype 1, 9, 12, 21 and 23 out of 40 viral isolates. The prevalence of different serotypes in different countries is presented in Table 4. Shi *et al.*

Table 2. Distribution of bluetongue serotypes in African continent

Country	Bluetongue virus serotypes on the basis of virus isolation	Bluetongue virus serotypes on the basis of serology (additional)	Reference
Egypt	1, 12, 16	4, 10	Barsoum (1992), Hafez and Ozawa(1981)
Morocco		10	Tomori *et al.* (1992)
Cameroon	1, 4, 5, 12, 14, 16	-	Ekue *et al.* (1985a, b)
Nigeria	1,2,3, 5-8, 10, 11, 12, 16, 22	4, 9, 13,20	Sellers (1984), Hermiman *et al.* (1983), Lee *et al.* (1974)
Senegal		6, 14	Lefevre and Taylor (1983)
Kenya	1, 2, 3, 4, 8, 13, + 3 untyped	9 others	Sellers (1984), Davies (1978)
Sudan	1, 2, 4, 5, 16	6-10, 14, 17, 20, 21, 22	Mohammed and Mellors (1990), Abu-Elzein (1985), Elfatih *et al.* (1987)
Malawi	-	1, 2, 3, 5, 8, 10, 14, 15, 20, 21, 22	Haresnape *et al.* (1988)
South Africa	1-15, 18, 19, 24	-	Sellers (1984), Nevill *et al.* (1992)
Zimbabwe	11	-	Blackburn *et al.* (1985)
Reuniun	2	4	Barrem *et al.* (1985)

Table 3. Distribution of bluetongue in Asia and Middle East

Country	Current distribution	Reference
China	Localized	Chen *et al.* (1996)
Cyprus	Widespread	OIE (1999)
Indonesia	Widespread	OIE (1999) Sendow *et al.* (1986)
Israel	Localized	Braverman and Galun (1973)
India	Widespread	Mehrotra (1992), OIE (1999), Prasad (2000)
Iraq	Present	Hafez *et al.* (1978)
Iran	Present	OIE (1999)
Jordan	Present	OIE (1999)
Japan	Present	Gard (1996)
Malaysia	Widespread	Hassan (1992)
Oman	Widespread	Hassan (1992)
Pakistan	Present	Hawkes (1996)
Saudi Arabia	Widespread	Abu *et al.* (1992)
Singapore	Localized	Personal communication (2003)
Syria	Widespread	Taylor *et al.* (1985)
Thailand	Widespread	Apiwatnakorn *et al.* (1996)
Turkey	Widespread	Burgu *et al.* (1992)
Yemen	Present	Stanley (1990)

Table 4. Distribution of bluetongue serotypes in Asia

Country	Bluetongue virus serotypes on the basis of virus isolation	Bluetongue virus serotypes on the basis of serology (additional)	Reference
Iran	-	3 (7, 20, 22)	Moakhar *et al.* (1988)
Israel	2, 4, 6, 10, 16		Hassan (1992), Sellers (1975)
Jordan	-	6 (2 ?, 4, 9, 13, 15)	Taylor *et al.* (1985), Taylor and Mellors (1994)
Oman	-	3, 4, 6, 14, 15, 17, 19, 20, 21, 22	Al-Busaidy and Mellors (19 91), Taylor *et al.* (1991)
Soudi Arabia	-	6, 14, 17, 19, 20	Hafez and Talor (1985)
Syria	2, 4, 6, 9, 13, 15		Taylor *et al.* (1985), Talor and Mellor (1994)
India	1, 2, 3, 4, 8, 9, 12, 16, 17, 18, 23	5, 6, 7, 10, 11, 13, 14, 15, 19, 20	Prasad (2000)
Pakistan	16	-	Ritter and Roy (1988)
China	1, 2, 3, 4, 12, 15, 16	-	Zhang *et al.* (1999)
Indonesia	1, 7, 9, 12, 21, 23	-	Sendow *et al.* (1993)
Japan	-	1, 12, 20	Miura *et al.* (1982)
Malaysia	1, 2, 3, 9, 16, 23	5, 20, 21	Hassan (1992), Sharifah *et al.* (1995)

(1992) have reported in China that serologically 47 animals were positive for BTV antibodies out of 1,616 animals imported from neighbouring countries, which were kept under quarantine.

Australia

It is widely considered that BTV entered Australia via infected insects carried in the summer monsoon from Indonesia. BTV was first identified in Australia in 1977 and the serotype involved was BTV 20. So far clinical BTV has not been reported from Australia in sheep, although BTV has been isolated from insect vector. In addition, seroconversion has also been recorded in indigenous cattle population in the endemic areas of the continent. So far eight serotypes i.e., BTV 1, 3,9, 15,16,20,21 and 23 have been isolated from insects or sentinel cattle in Australia (Doyle 1992) (Table 5). In view of BTV establishment in Australia, a BTV research management committee was established in early 1989 to supervise the existing BTV research program and the development of new projects. These projects covered insect studies, field epizootiological studies, vaccine development and evaluation of improved diagnostic techniques for not allowing the virus to establish and spread infection to Australian livestock.

Table 5. Distribution of bluetongue virus serotypes in Oceania

Country	Bluetongue virus serotypes on the basis of virus isolation	Bluetongue virus serotypes on the basis of serology	Reference
Australia	1, 3, 9, 15, 16, 20, 21, 23	-	Doyle (1992)
Papua New Guinea	-	1, 21, 23	Gard *et al.* (1985)

Europe

The first evidence of prevalence of BTV in European continent came from Cyprus where the disease similar to BT was described in sheep in 1943. Subsequently, BTV was noticed in other countries in the same region. An epizootic of BT was reported from Portugal in 1957 by Manso-Ribeiro and co-workers (1957) and Spain (Lopez and Botija 1958) during 1956-57. BTV was also recorded in Greek Island of Lesbos during 1979-80. An extensive survey of BTV antibodies during 1986-89 of southern Turkey indicated an active infection of BTV in both cattle and sheep in some of the provinces. The per cent positivity varied from 10.8% to 48.5% in sheep of different provinces of southern Turkey. A high percentage of cattle sera (52%) were positive in Adana province while neighbouring province of Mersin showed 31% positivity to BTV antibodies. A high per cent positivity in sheep could be attributed to vaccination program undertaken in sheep; however, the co-existence of BTV antibodies in cattle sera, which were never vaccinated against this virus confirms the occurrence of BTV infection in Turkey. The most prevalent serotype in Turkey appeared to be BTV 4 (Burgu *et al.* 1992). After the outbreak of BTV infection in Lesbos during 1979-80, a policy was strictly implemented for early detection of BTV positive cases and restriction on movement of animals (Papadopoulos 1992). A major epidemic of BT occurred in nothern and eastern Greece and islands in eastern Aegean Sea in 1999. No clinical case of BT was recorded in 2000 in Greece. However, several BT suspected cases recorded in sheep flocks of Ioannina and Grevena in Greece. The suspected cases were characterised by high fever, lameness, face oedema with nasal discharge, inflammation of buccal cavity and lips. Morbidity and mortality rates were 3.5 % and 1 %, respectively. It is not clear whether the BT cases were due to over-wintering of BTV previously recorded in Greece or new incursion? The severity of infection and high prevalence rate suggested that the animals were not previously exposed to the virus (OIE 2001). It is yet to be investigated how BTV infection reached there. Was it due to import of viraemic animals or wind-borne infective vectors carried from neighboring countries? These questions have not been conclusively resolved.

Outbreaks of BT also occurred in different provinces of Italy for the first time and more than 100 animals have been reported dead in these outbreaks in 2001. Similarly outbreaks of BT have been reported from Macedonia of former Yugoslav republic in 2001. The virus isolation efforts

were successful and serotype 4 and 9 suspected. Another serotype 2 was also suspected however, results were not confirmed. These reports and previous reports of prevalence of BTV in Bulgaria suggest that there is competent vector in some parts of Europe and risk of transmission of BTV to those countries which have never witnessed BT is very high (OIE 2002). The prevalence of different serotypes of BTV are summarized in the Table 6 and 7.

Table 6. Distribution of bluetongue in Europe (Centre for Emerging Issues 2004)

Country	Reported outbreaks, year
Bulgaria	1999, 2001, 2002
Greece	1998, 1999, 2000, 2001
Turkey	1999, 2000
Serbia and Montenegro	2001, 2002
Former Yugoslav Republic of Macedonia	2001, 2002, 2003
Croatia	2001, 2004
Italy	2000, 2001, 2002, 2003
Spain	2000, 2003
France	2000, 2001, 2003, 2004
Albania	2002
Bosnia and Herzegovina	2002, 2003
Cyprus	1998, 1999, 2000, 2001, 2002, 2003, 2004

Table 7. Distribution of bluetongue serotypes in Europe

Country	Bluetongue virus serotypes on the basis of virus isolation	Bluetongue virus serotypes on the basis of serology (additional)	Reference
Cyprus	3 (pre-1965) 4 (1969, 1977) 16	10, 12	Ritter and Roy (1988), Polydorou (1978)
Greece (Lesbos, Rhodes)	4 (1979) 1, 16	-	Papadopoulos (1992)
Portugal	10 (1957-59)	-	Sellers (1984)
Spain	10 (1957-60)	-	Sellers (1984)
Turkey	4 (1977-80), 16	2 (1981)	Burgu *et al.* (1992)
Italy	4, 16	-	CEI, 2004
France	4, 16	-	CEI, 2004

North America

North American continent is mainly composed of USA and Canada. Prevalence of BTV in USA is quite wide-spread particularly in southern states. A clinical syndrome as sore muzzle of sheep was reported way back in 1952 from Texas and in 1953 from California. Lateron BTV was isolated from affected sheep and the presence of BT in USA was confirmed. The possible mode of transmission of BTV to USA is suspected to be through import of viraemic animals. However, this is speculative. Subsequently prevalence of BTV was reported in wild and domestic ruminants in other

states. Bluetongue virus serotype 10 was for the first time isolated in USA (Mckercher *et al.* 1953). Subsequently BTV serotype 11 (1955), serotype 17 (1962) and serotype 13 (1967) were isolated (Barber 1979). Now the disease is prevalent in domestic and wild ruminants in the endemic form in several states of USA. Since there was no restriction on movement of animals, except on animals showing clinical symptoms, the BTV spread widely all over USA (Table 8).

Table 8. Distribution of bluetongue serotypes in North America

Country	Bluetongue virus serotypes on the basis of virus isolation	Bluetongue virus serotypes on the basis of serology (additional)	Reference
Canada	11	-	Dulac *et al.* (1992), Shapiro *et al.* (1987), Thomas *et al.* (1982)
Mexico	10, 11, 13, 17	-	Stott *et al.* (1989)
USA	2, 10, 11, 13, 17	-	Gibbs and Greiner (1994), Heidner *et al.* (1992)

A wide variety of wild free-ranging animal species have been found serologically positive for BTV infection. These species included bighorn sheep, barbary sheep, elk, moose and pronghorn antelope. It has been suggested that BTV was the major factor in disappearance of bighorn sheep from west Texas. Because bighorn sheep populations declined appreciably at the time BTV was a rampant disease among domestic sheep on the same range (Robinson *et al.* 1967). In 1976, thousands of pronghorn antelope and mule deer died due to BTV in Wyoming, Colorado, Nebraska and New Mexico. In south Texas, where BTV is enzootic, antibody prevalence was as high as 89% in adult white tailed deer (Hoff and Trainer 1974, Marburger *et al.* 1970). This indicated a very high prevalence of BTV in white-tailed deer in Texas. Additional losses were suggested to occur *in utero* when BTV transmission and the deer gestation period overlap. Experimental infection of BTV during first trimester of gestation in white-tailed deer resulted in early absorption or uncomplicated abortions (Thomas and Trainer 1970). These observations clearly suggested that BTV represents a potential threat to the white-tailed deer populations.

The climatic conditions are highly variable from north to south and east to west which is an important factor regulating the spread of vector and thus BTV infection. *Culicoides variipennis* has been identified as a predominant vector of BTV transmission. Since frost free days are even less than 100 days in northern and north-eastern states, BTV could not establish itself in this region. However, the disease has a gradual increase in its presence from central states to southern states of USA because frost free days are increasing from more than 150 -200 days (Johnson 1992).

Approximately 30% of the herds or flocks from which virus was isolated had more than one serotype. Serological monitoring was done in Canada during 1969 to establish the introduction of BTV in this country through imported animals from USA. During a survey of nearly 10,000 cattle imported from USA, 261 sera were positive in complement fixation (CF) test. The testing of 524 native Canadian animals which were in contact with the seropositive American cattle, revealed an additional 221 seropositive animals. After slaughtering seropositive animals, Agriculture Canada declared the country infected with BTV and established a quarantine zone.

South America and Caribbean

South American countries Surinam and Guyana were found serologically positive for BTV 6,14, and 17. Limited prevalence studies were conducted in Peru, Chile and Equador (Homan *et al.* 1992). Isolation of BTV 4 was made from Brazilian bulls. The prevalence of BTV infection in French Guyana is also documented. Similarly, presence of BTV infection in ruminant livestock in Antigua, Barbados, Grenada, Jamaica, St. Kitts, St. Lueis, Trinidad, Tobago, Costa Rica and Northern Colombia is on record. Serological studies revealed widespread distribution of BTV 1, 6, 12, 14 and 17 in these islands. Some serotypes were circulating in Costa Rica and Colombia. Serological evidence for presence of BTV in Mexico was demonstrated in 1982. Isolation of BTV 10, 11, 13 and 17 is well documented from feedlot cattle from northern Mexican states. The BTV distribution and isolates from South America, Caribbean countries are shown in Tables 9-12.

Bluetongue virus infection in India

Indian climates as well as the domestic and wild animal species are very diverse and suitable for perpetuation of *Culicoides* vectors as dealt in detail under the Chapter 6 – *Vector*. The serological surveys conducted in different geographical locales during past 30 years have demonstrated extensive prevalence of BTV antibodies in different species of animals (Fig. 11).

Table 9. Distribution of bluetongue in Caribbean

Country	Current distribution	Reference
Antigua and Barbuda	Widespread	Homan *et al.* (1992)
Barbados	Widespread	OIE (1999)
Bahamas	Widespread	Gibbs and Greiner (1985)
Dominican Republic	Widespread	OIE (1999)
Grenada	Widespread	Homan *et al.* (1992)
Guadeloupe	Present	OIE (1999)
Jamaica	Widespread	Thompson *et al.* (1992)
Saint Kitts and Nevis	Widespread	Homan·*et al.* (1992)
Saint Lucia	Widespread	Homan *et al.* (1992)
Puerto Rico	Widespread	Thompson *et al.* (1992)
Trinidad and Tobago	Widespread	Thompson *et al.* (1992)

Table 10. Distribution of bluetongue in Central America

Country	Current distribution	Reference
Costa Rica	Widespread	Thompson *et al.* (1992)
Guatemala	Widespread	Thompson *et al.* (1992)
Honduras	Widespread	Thompson *et al.* (1992)
Nicaragua	Widespread	Thompson *et al.* (1992)
Panama	Widespread	OIE (1999)
El Salvador	Widespread	Thompson *et al.* (1992)

Table 11. Distribution of bluetongue in South America

Country	Current distribution	
Argentina	Present	OIE (1999)
Brazil	Present	Hawkes (1996), Melo *et al.* (2000)
Chile	Present	Tamayo *et al.* (1985)
Colombia	Localised	Homan *et al.* (1992)
French Guiana	Widespread	Lancelot *et al.*(1989)
Guyana	Widespread	Gibbs and Greiner (1985)
Peru	Widespread	Rosadio *et al.* (1984)
Paraguay	Present	OIE (1999)
Surinam	Widespread	Gibbs and Greiner (1985)
Venezuela	Present	OIE (1999)

Table 12. Distribution of bluetongue serotypes in South America and Caribbean

Country	Bluetongue virus serotypes on the basis of virus isolation	Bluetongue virus serotypes on the serotypes on the (additional)	Reference
Costa Rica	1,3 , 6	-	Mo *et al.* (1994)
El Salvador	1, 3, 6	-	Mo *et al.* (1994)
Guatemala	1, 3, 6, 17	-	Mo *et al.* (1994)
Honduras	1, 2, 6, 17	-	Mo *et al.* (1994)
Nicaragua	1, 3, 6	-	Mo *et al.* (1994)
Panama	1, 3, 6	-	Mo *et al.* (1994)
Barbados	1, 3	-	Mo *et al.* (1994)
Dominican Republic	4, 6, 8	-	Mo *et al.* (1994)
Jamaica	3, 12	-	Mo *et al.* (1994)
Puerto Rico	3, 4, 17	-	Mo *et al.* (1994), Shaw (1992)
Trinidad and Tobago	3	-	Mo *et al.* (1994)
Carabbean Islandsa	-	1, 6, 12, 14, 17	Mo *et al.* (1994)
Brazil	4	-	Gurgel-da-Cunha (1990), Grocock and (1990), Grocock and
Colombia	-	1, 6, 12, 14, 17	Homan *et al.* (1992)
Surinam	-	6, 14, 17	Homan *et al.* (1992)
Guyana	-	6, 17, 17	Homan *et al.* (1992)

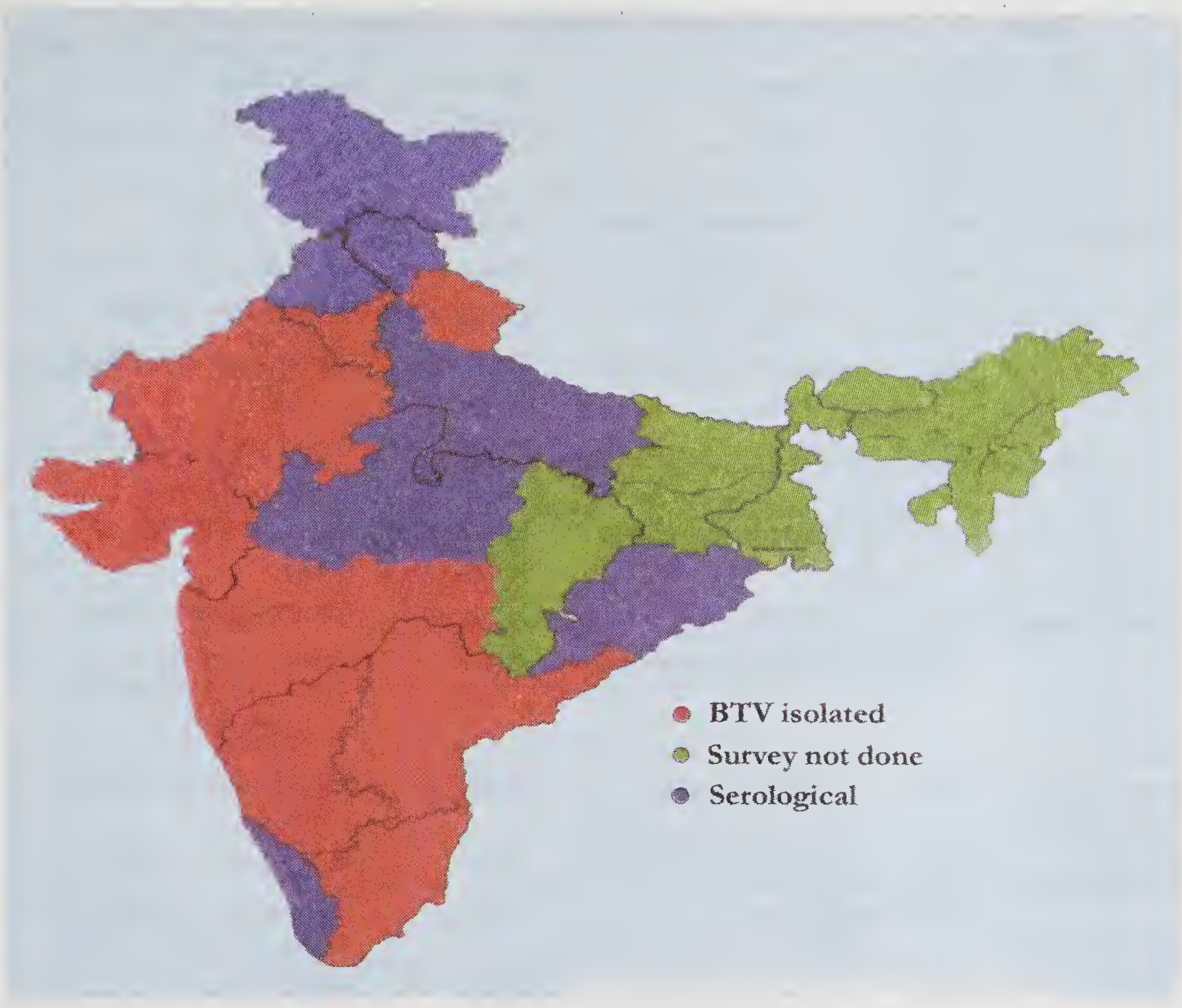

Fig. 11. Distribution of BTV in India

Sheep

The first outbreak of BTV in India was recorded in 1964 among sheep and goats in Maharashtra State, on the basis of clinical signs and detection of BTV antibodies in the sera of animals which had recovered (Sapre 1964). However, BTV could not be isolated due to a lack of the necessary infrastructure. It is interesting to note that the disease was also observed in indigenous sheep and goats, which are said to be comparatively resistant to severe BTV infection. Subsequently Bhambani and Singh (1968) recorded an outbreak of BTV in sheep in a Government Livestock Farm in Uttar Pradesh and claimed to have isolated BTV by successfully transmitting the disease to experimental sheep. Since the initial detection of BTV in sheep and goats in Maharashtra in 1964, serological evidence of the infection has been reported from almost all regions of India. The outbreaks occurred till 1992 are presented in Table 13.

In the Tathawada Exotic Breed Farm, Pune, and Maharashtra BTV was observed between 1967 and 1970 . The virus serotypes 1 and 16 were isolated in cell culture, while serotypes 2, 7,9 and 10 were detected on the basis of serum neutralising antibodies. The breeds of sheep affected were Southdown, Rambouillet, and Southdown × Bannur crosses (Vasudevan 1982). In 1973, an outbreak of BTV was recorded in Russian Merino in Kothipura Farm, Himachal Pradesh (Uppal and Vasudevan 1980). In 1975,

Table 13. Outbreaks of clinical bluetongue in different states of India

State	Month/ Year	Breed/ species	District/ region	Reference
(1)	(2)	(3)	(4)	(5)
Maharashtra	1964	Indigenous sheep	Pune	Sapre (1964)
	1967-70	Exotic sheep	Pune	Mongha *et al.* (1970)
	1973	Exotic sheep	Dhulia	Vasudevan (1982)
	1982	Exotic, crossbred sheep	Pune, Dhulia	Harbola *et al.* (1982)
	1982	Indigenous sheep	Marthwada region	Singh *et al.* (1982)
	1981-1983	Indigenous sheep	Marthwada region	Kulkarni and Kulkarni (1984)
Uttar Pradesh	1967	Sheep	Mathura	Bhambani and Singh
	1987	Indigenous sheep	Rapur	(1967), Mehrotra *et al.* (1989), Mehrotra *et al.* (1995)
Himachal Pradesh	1973	Exotic sheep	Bilaspur	Uppal and Vasudevan (1980)
Haryana	1975	Exotic	Hisar	Vasudevan (1982)
	1985-1988	Exotic	Hisar	Jain *et al.* (1986), Mahajan *et al.* (1991)
	1999	Indigenous sheep	Sirsa	Malik (2000)
Karnataka	1982	Indigenous sheep	Bidar, Gulbarga	Srinivas (1982)
Madhya Pradesh	1996	Sheep	Khanduva	Mehrotra *et al.* (1996)
Jammu & Kashmir	1996	Sheep	Srinagar	Mehrotra *et al.* (1996)
Rajasthan	1983	Exotic, indigenous sheep	CSWRI, Tonk	Lonkar *et al.* (1983)
	1985	Exotic, indigenous, crossbred sheep	CSWRI, Tonk	Sharma *et al.* (1985)
	1992-95	indigenous, crossbred and indigenous	CSWRI, CSWRI, Chitaurgarh, Fatehpur, Chiru and Jaipur	Prasad and Srivastava (1995)
Andhra Pradesh	1985	NM	Kurnool	Haribabu (1985)
	1983-85	I,E &CB	Mamnoor, Mahbubnagar, Mamidipally	Mullick (1988)
	1994	Indigenous sheep	Krishna, Khamam, Warangal, Nizamabad,	Dr. Y. Gopal Setty and M.A. Khan (Personal communication)

(Table 13. *Concluded*)

(1)	(2)	(3)	(4)	(5)
			Nellore, Gunture, Chittoor, Karimnagar, Adilabad	
Gujarat	1993	Exotic sheep	Rajkot	B. Singh (personal communication)
Tamil Nadu	1991	Indigenous sheep	Central	Janakiraman *et al.*
	1991	Indigenous sheep Shivaganga	districts	(1991) Mehrotra *et al.* (1991) Mehrotra *et al.* (1996)
	1992	Indigenous sheep	Shivaganga Taluk	Nachimuthu *et al.* (1992)

an outbreak of BTV was recorded in the Central Sheep Breeding Farm (CSBF), Hisar. The Animal Virus Research Institute in Pirbright, United Kingdom, was able to isolate BTV serotypes 1 and 4 from the clinical material sent to the institute (Vasudevan 1982). The breed of sheep affected in this outbreak was Corriedale.

In a serological survey conducted by Sodhi *et al.* (1981) in Punjab State, 6.64% sheep were positive for BTV antibodies by the immunodiffusion test. The prevalence of BTV antibodies was higher in exotic breeds than that in indigenous animals. Srinivas *et al.* (1982) reported an outbreak of BTV in sheep and goats in Bidar, Gulbarga and nine other districts of Karnataka State, where about 50% of the population of these animals were affected. A very severe outbreak occurred in eastern Maharashtra adjoining the Telangana region of Andhra Pradesh, which later spread to western Marathawada, affecting sheep in all districts of the region. The morbidity was as high as 80% in village flocks (Singh *et al.* 1982). In 1982, Harbola *et al.* (1982) attended an outbreak of BTV in Maharashtra. Attempts to isolate the virus were unsuccessful. The authors reported that crosses of indigenous Chokla breed with Merino were more susceptible than pure Chokla breed. However, Deccani sheep (indigenous breed) showed clinical signs during the outbreak. Of 8, 980 exotic, crossbred and indigenous sheep, 868 were affected, 100 of which died, giving an overall case fatality rate of 11.52%. Lonkar *et al.* (1983) observed an outbreak of BTV in sheep at the Central Sheep and Wool Research Institute (CSWRI) in Avikanagar, Rajasthan, and reported that Rambouillet and Merino were more susceptible than indigenous breeds. The morbidity rate in Merino and Rambouillet was 33.3% and 23.5% respectively. Mortality in adult Merino was 35.3%. Bandyopadhyay and Mullick (1983) reported BTV antibodies in sheep sera obtained from Haryana, Uttar Pradesh, Rajasthan and Andhra Pradesh. The incidence of BTV antibodies was higher in exotic sheep than in indigenous and cross-bred animals. Similarly Mehrotra and Shukla (1984), detected BTV

antibodies in sheep sera obtained from Maharashtra, Andhra Pradesh, Karnataka, Rajasthan, Jammu and Kashmir and Himachal Pradesh.

Sharma *et al.* (1981) conducted a systematic epizootiological study of BTV at the CSWRI. They reported 15.3% fatality due to this infection. They also monitored the presence of BTV antibodies in exotic sheep of various breeds including Merino, Rambouillet, Dorset, Suffolk, Karakul, crossbreds and indigenous breeds. They concluded that exotic breeds of sheep were more susceptible to BTV infection than indigenous breeds. The species and breeds susceptibles to BTV are depicted in Table 14.

Table 14. Different species and breeds of domestic ruminants reported to be susceptible to bluetongue virus infection in India

Species	Breeds	Clinical BT recorded	inapparent infection recorded by serology
(1)	(2)	(3)	(4)
Exotic sheep	Corriedale	+	+
	Dorset	+	+
	Rambouillet	+	+
	Russian Merino	+	+
	Southdown	+	+
	Suffolk	+	+
	Karakul	-	-
Crossbred Sheep	Southdown × Bannur	+	+
	Rambouillet × Nali	?	+
	Rambouillet × Sunali	?	+
	Deccan × Merino	+	+
	Chokla × Merino	+	+
	Rambouillet × Pole Dorset		
	Hisardale × Nali		
		?	+
Indigenous sheep	Nali	-	+
	Sonali	-	+
	Chokla	-	+
	Deccan	+	+
	Malpura	-	+
	Ramanathapuram	?	+
	Vellai	?	+
	Kilakarashal	?	+
	Mandia	?	+
	Mecheri	?	+
Goat	Goat (undescript)*	+	+
	Gaddi goat	-	+
	Alpine goat	-	+
	Sirohi	-	+
	Nagauri	-	+
Crossbred goat	Alpine × Sirohi	-	+
	Tokanburg × Sirohi	-	+
Indigenous	Hariana	-	+

(Table 14. *Concluded*)

(1)	(2)	(3)	(4)
cattle	Sahiwal	-	+
	Rathi	-	+
	Red sindhi	-	+
	Tharparker	-	+
Exotic cattle	Jersey	-	+
	Holsteine Frasien	-	+
	Reddane	-	+
Crossbred cattle	Karanswiss × Friesian	-	+
	Rathi × Jersey	-	+
	Rathi × Reddane	-	+
	Jersey × Red Sindhi	-	+
	Friesian × Sahiwal	-	+
	Reddane × Sahiwal	-	+
	Sahiwal × Jersey	-	+
	Tharparker × Jersey	-	+
	Tharparker × Friesian	-	+
	Hariana × Friesian	-	+
Buffalo	Murrah	-	+

Kulkarni and Kulkarni (1984) claimed to have isolated BTV serotypes 8 and 18 from sheep affected with BTV in developing chicken embryos. Haribabu (1985) conducted haematological studies on sheep naturally infected with BTV reported a marked decrease in the total leukocyte count in the affected sheep. Similar observations have been made in sheep experimentally-inoculated with BTV serotype 1 (Chander *et al.* 1990).

Jain *et al.* (1986) reported a severe outbreak of BTV in Rambouillet sheep three months after their arrival from the USA at the CSBF, Hisar. BTV serotype 1 was isolated in 10 to 12 day-old chicken embryos and BHK-21 cell cultures from the blood of affected sheep. The disease was transmitted to the crossbred sheep experimentally, using infective material from sheep affected in this outbreak. In 1986, a serological survey of BTV antibodies was conducted in the sheep, goat, cattle, buffalo, camel and horse populations in and around Hisar, Haryana. The results indicated the presence of BTV antibodies in 82.2% of exotic ewes which had aborted, 36.6% of apparently healthy exotic ewes and 14.3% of exotic rams. The prevalence of BTV antibodies was lower in indigenous sheep than that in exotic breeds. However, the authors failed to detect BTV antibodies in camels, horses, goats and buffaloes (Prasad *et al.* 1987). Dubey *et al.* (1987) reported the presence of BTV antibodies among sheep at the CSWRI, Rajasthan. BTV serotype 1 was isolated from sheep in CSWRI, Avikanagar, Rajasthan (Prasad *et al.* 1994) besides these two northern and western states, another serological survey of BTV infection was conducted in four organised sheep farms of Andhra Pradesh in south India. Here, overall morbidity was reported as 21.5%; morbidity in crossbred sheep was higher than that in exotic and indigenous breeds (Mullick 1988).

Between 1985 and 1988, another epizootiological study of BTV was carried out in the CSBF in Haryana (Mahajan *et al.* 1991). The study

revealed a case fatality rate of 31.2% in 1985, which increased to 42.7% in 1986. In 1987 and 1988 the mortality rate fell to 7.0% and 7.4% respectively.

In a retrospective study at the CSWRI between 1980 and 1987, Srivastava *et al.* (1989) correlated congenital defects in lambs with BTV infection. Among the rare deformities recorded in various breeds of sheep were suicephaly, presence of two lateral oral openings, bifid tongue, and absence of various parts of the body, such as the pelvic girdle, rectum, sex organs, hind legs, abdominal muscles and skin. Since the incidence of BTV in the flocks at the CSWRI coincides with the breeding cycle and the presence of this disease has been confirmed on the farm. The study indicated the possible role of BTV in congenital lamb defects. Our recent *in vitro* studies on susceptibility of cattle and sheep lymphocytes and monocytes to BTV 1 infection have suggested that the replication of the virus occurs in these cells (Garg and Prasad 1994, Garg and Prasad 1995, Kumar and Prasad 1996).

Goats

The first evidence of BTV in goats was recorded by Sapre (1964) in Maharashtra. Although typical BT has not been reported, there are several reports of the presence of BTV antibodies in goats in several states. Sodhi *et al.* (1981) reported a 1.4% incidence of BTV antibodies in goats in Punjab State, while Bandyopadhyay and Mullick (1983) reported a 3.0% prevalence of BTV antibodies in goats in Uttar Pradesh. In a recent serological survey carried out at the Western Regional Station of the Central Goat Research Institute in Avikanagar, Rajasthan 33.3% of goat sera were positive for BTV antibodies by the AGID test (N C Jain and G Prasad, unpublished data). In another recent serological survey conducted in Hisar, Haryana, 40 goat serum samples were tested, of which 14 (35%) were positive for BTV antibodies. However, clinical BTV disease was not observed in these goats. The role of goat populations in the maintenance of BTV in nature is not known.

Cattle and buffalo

The earliest evidence of BTV infection in bovines in India is documented in the 1978 Annual Report of the Indian Veterinary Research Institute (IVRI). According to this report, 3.7% of cattle sera were positive for BTV antibodies. Sharma *et al.* (1985) conducted a serological survey in cattle and buffalo in Punjab State and recorded the presence of BTV antibodies in 6.8% of cattle sera. Oberoi *et al.* (1988) demonstrated the presence of BTV antibodies in 38.5% of buffalo sera in Punjab State. They also recorded 70% of cattle sera positive for BTV antibodies. Tongaonkar *et al.* (1983) reported that in Gujarat State, 13.4% of buffalo and 15.6% of cattle were positive for BTV antibodies. The positive sera revealed presence of antibodies against serotypes 1, 15 and 17. Bandyopadhyay and Mullick (1983) reported that 3.7% of cattle sera positive for BTV antibodies. Subsequently, Mehrotra and Shukla (1984) tested serum samples from seven states (Andhra Pradesh, Karnataka, Gujarat, Punjab, Orissa, Himachal

Pradesh and West Bengal). A total of 154 cattle sera were tested, of which 28 (18%) were positive for BTV antibodies.

In 1990, an extensive survey of BTV antibodies in cattle and buffalo was conducted in Haryana State. Serum samples were collected from 549 cattle and 498 buffalo, of which 4.2% and 10.6%, respectively, were positive (Jain *et al.* 1992). No clinical BTV has been observed in cattle and buffalo, but these animals may play an important role in the maintenance of the virus in nature. The presence of BTV antibodies in cattle and buffalo has become a serious impediment to the export of the germplasm of these animals. In an extensive serological survey of BTV infection in cattle and buffaloes in northern India, a quite high prevalence of BTV antibodies has been recorded (Srivastava *et al.* 1995). These reports established that BTV infection is widely present in cattle and buffalo herds in India.

Other animals

The presence of BTV antibodies has been reported in several wild ruminants from other countries of the world. However, no systematic survey has yet been conducted to assess the status of BTV infection in Indian wildlife. Only one elephant serum sample tested was found positive for BTV antibodies. One serum sample from black buck was found negative for BTV antibodies. In a separate study, none of the 128 camel sera samples was found positive for BTV antibodies (Prasad *et al.* 1987), although the presence of BTV antibodies in camel has been demonstrated in some other countries (Barzilai 1982). However, in one of our recent serological surveys of camel sera from Rajasthan, suggested presence of antibodies to BTV (Malik *et al.* 2002).

A study was conducted with the collaboration of Wildlife Institute of India, Dehradun, in and around Sariska Tiger Reserve, Rajasthan, to determine whether there is transmission of BTV from domestic to wild and vice versa. Our study suggested that since 2 sambar (wild ruminant) and several goat and cattle samples were positive for BTV antibodies, there is real possibility of both ways transmission of BTV in the wild life reserve where domestic and wild ruminants intermix for grazing in the interface conflict situation (Prasad *et al.* 1998).

BTV serotypes reported from India

The segmented and dsRNA genome of BTV makes it prone to frequent mutations and genetic reassortments leading to the emergence of a new serotype or antigenic variant of the same serotype. To date, 24 serotypes of BTV have been recorded world-wide, 21 of which have been reported in India (Table 15-17).

Table 15. Total number of bluetongue virus serotypes detected in India

Basis	Serotypes	Total
Virus isolation	1, 2, 3, 4, 8, 9, 12, 16, 17, 18, 23	11
Neutralising antibodies (additional)	5, 6, 7, 10, 11, 13, 14, 15, 19, 20	10
	Total	21

Table 16. State-wise prevalence of different serotypes of bluetongue virus

State	Serotype of bluetongue virus on the basis of neutralizing antibodies	Serotype of bluetongue virus on the basis of virus isolation	Total
Jammu & Kashmir	-	18	1
Himachal Pradesh	4	3, 9, 16, 17	5
Haryana	2, 8, 12, 16	1, 4	6
Rajasthan	-	1	1
Uttar Pradesh	23	9, 18, 23	3
Madhya Pradesh	18	18	1
Gujarat	1, 2, 3, 5, 8, 9, 10, 11, 12, 13, 15, 16, 17, 20	6	15
Maharashtra	2, 3, 4, 5, 6, 7, 8, 10, 12, 13, 16	1, 9, 16, 18, 4, 17	17
Andhra Pradesh	4, 6, 12, 13, 14, 17, 18, 19	2	9
Karnataka	1, 4, 16	18	4
Tamil Nadu	1, 3, 4, 5, 6, 7, 11, 12, 14, 15,16 17, 19, 20	3, 16	14

Uppal and Vasudevan (1980) reported the presence of BTV serotypes 3, 4, 9, 16 and 17 in Russian Merino sheep affected in two outbreaks at Kothipura Farm in Himachal Pradesh. BTV serotypes 1 and 4 were

Table 17. Serotype-wise prevalence of bluetongue virus in different states of India

States	Serotype
Haryana,Rajasthan, Gujarat,Maharashtra, Karnataka, Tamil Nadu	BTV1
Haryana,Maharastra, Gujarat,Andhra Pradesh (AP)	BTV2
Himachal Pradesh (HP),Gujarat,Maharashtra,Tamil Nadu (TN)	BTV3
Haryana,HP, Maharashtra, Karnataka, TN, AP	BTV4
Gujarat, Maharashtra, TN	BTV5
Gujarat, Maharashtra, TN	BTV6
Maharashtra, TN	BTV7
Haryana,Gujarat, Maharashtra	BTV8
HP, Uttar Pradesh (UP), Gujarat, Maharashtra	BTV9
Maharashtra, Gujarat	BTV10
TN, Gujarat	BTV11
Haryana,Maharashtra, Gujarat,AP, TN	BTV12
Maharashtra,Gujarat, AP	BTV13
AP, TN	BTV14
Gujarat, TN	BTV15
Haryana, HP, Gujarat, Maharashtra,Karnataka, TN	BTV16
HP,Gujarat, Maharashtra,TN, AP	BTV17
HP, Gujarat, MP, J & K, Maharashtra, AP	BTV18
AP, TN	BTV19
Gujarat, TN	BTV20
UP	BTV23
Not reported	BTV21,22, 24

incriminated in a BTV outbreak in Australian Corriedale Sheep at the CSBF, Hisar (Uppal and Vasudevan 1980). Subsequently, serum samples sent to Onderstepoort by Sriguppi (Sriguppi 1982) and Choudhary (1982) from Maharashtra State showed the presence of antibodies against BTV serotypes 1, 2, 3, 4, 7, 10, 16 and 17.

Kulkarni and Kulkarni (1984) reported the isolation of BTV serotypes 9 and 18 from two outbreaks (in 1981 and 1983) in Maharashtra. In 1985, BTV serotype 1 was isolated from Rambouillet sheep affected with BTV at the CSBF, Hisar (Jain *et al.* 1986). Subsequently, Jain *et al.* (1988) isolated BTV from a *Culicoides* sp. of midge collected from the same farm. However, the serotype of this isolate could not be ascertained. Prasad *et al.* (1994) isolated serotype 1 from sheep in CSWRI, Avikanagar, and Rajasthan. Mehrotra *et al.* (1989) isolated BTV from Maharashtra which was subsequently typed as BTV 18. Mehrotra *et al.* (1996) reported isolation of BTV from Madhya Pradesh, Tamil Nadu, Jammu and Kashmir and Uttar Pradesh. Mehrotra *et al* (1995) reported concurrent outbreaks of BT and PPR and isolated BTV type 23.

Modes of transmission of the virus

Vectors: Culicoides midges are proven vectors of BTV. Based on the dispersal of the vectors, BTV is distributed in America, Africa, Middle East, Indian-sub-continent, South East Asia, parts of Australia and parts of Europe. *Culicoides* midges generally breed in hot and humid climate particularly in the edges of water channels, ditches and wherever water is accumulated in the farm. The insects are totally dependent on blood meal of the animals. Hence when they bite the animals carrying BTV in the blood, they pick the virus along with blood meal. Then the virus multiplies in the insect tissues and when such insects bite the healthy animals, they transmit the disease. *Culicoides* normally feed on animals during dusk and dawn.

Culicoides breeds in many habitats particularly in damp muddy areas, stagnant water and in cow dung. Moisture is indeed important for its life cycle but some species may also be found in arid areas and some of these *Culicoides* breed even in saline water. Female *Culicoides* take a blood meal every 3-4 days until the end of its life which is around 70 days. If the blood contains virus, it infects cells of haemocoel and salivary glands of the vector. After an incubation period of 7-10 days, the virus is excreted in saliva for further transmission to susceptible animals (Mellor 1990). There is no evidence of transovarian transmission in arthropods and not all species of *Culicoides* are vectors. Different species constitute the principal vector in different parts of the world. The presence of BTV infection in ruminants has been reported to be associated with rainfall in Sudan, Nigeria, Kenya, South Africa, the Caribbean and Indonesia.

The threshold of annual rainfall required for the persistence of BTV in an area has been estimated as between 750 and 100 mm (Walker and Davies 1971). The abundance and distribution of *Culicoides* sp. are also dependent on temperature, moisture and wind speed and its direction (Ward

1994). Long range wind - borne dispersal of infected *Culicoides* may some times occur and constitute a mechanism by which BTV can be introduced to a distant area. The metabolism of vector is dependent on temperature and warmth which is necessary for the hatching of eggs and development of larvae. *Culicoides* species are active and fly at temperature of 13° to 35° C and moisture is essential for the breeding sites, *Culicoides* species have been shown to be carried 5 to 6 km by winds and there is circumstantial evidence of the wind-borne spread of the vector up to 700 km (Homan *et al.* 1990).

Vertical transmission: Since the disease is non-contagious, vertical transmission of the virus from infected ruminants could be possible. However, reports about congenital transfer of the virus are not only incomplete but contradictory and confusing. In sheep Young and Cordy (1964) and Anderson and Jensen (1969) have shown that BTV crosses placenta and causes foetal damage, whereas, Flangan *et al.* (1982) were unable to demonstrate transplacental infection. Further Gibbs *et al.* (1979) showed that sheep foetuses infected in mid gestation were viremic at birth until 2 months of age while Richardson *et al.* (1985) could not reproduce this work. In addition, there is no convincing scientific evidence that natural BTV infection ever crosses the placenta to cause foetal infection and production of persistently infected adult animals. As such embryo transfer reduces the risk of transmission when compared with movements of breeding livestock (Gibbs and Greiner 1988). The risk of importing BTV by importation of live ruminant livestock from endemic countries is considered to be much greater than import of germplasm (Roberts *et al.* 1993).

Recent outbreaks of BT in several European countries have demonstrated that BTV is spreading to areas which were free of the disease. Global warming has been predicted to be faster than earlier predictions. Increase in environmental temperature would mean that the *Culicoides* vectors would be able to breed and thrive in the regions which were earlier hostile to the vector. The movement of animals and the competent vectors across the nations would increase chances of outbreaks of the BTV. Therefore, monitoring the movement of the potential vectors, infected ruminant animals and their products would be challenge for the governments and quarantine authorities. Serotype specific molecular typing assays would be required for effective monitoring and surveillance of different serotypes of the virus. It has been now confirmed that distinct strains of the virus (virus topotypes) vectored by different species of *Culicoides* vectors occur in specific regions of the world. Topotypes of BTV and *Culicoides* species that occur within each episystem are relatively stable, despite extensive and on going trade and movement of animals, their products and germplasm between the episystems. However, much remains to be understood about the ecological, climatic and environmental factors that lead to expansion of episystems as recently occurred in Europe. Therefore, it is important that these factors are clearly understood in relation to BTV and its vector.

Distribution of BTV in Asia and Europe is similar to that in North America and far beyond the 40^0 N limits that traditionally was proposed.

Recently BTV has occurred to approximately 40^0 N in Europe and BTV infection has been documented as far as 50^0 N in Asia. Very little is known about these northern Eurasian BTV episystems, both in terms of their vector species and BTV strains that occur within each. Similarly, the characterization of BTV strains, relative importance potential vector species/ strain that occur in variable portions of the extensive BTV episystems in South America, Africa, Middle East and Asia, are required.

The available diagnostic technologies are sufficient to specifically and sensitively detect the BTV infection in vector as well as vertebrate hosts. Thus, the global and regional distribution of BTV infection can now be comprehensively determined using appropriate surveillance and monitoring. The collation of such data should be an important international goal given that BTV has been identified in each continent except Antarctica, and that information is lacking from many regions of the world. An integrated, comprehensive network of surveillance, monitoring and reporting system is required to establish the global limits of the distribution of BTV and of competent *Culicoides* vectors.

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5

Bluetongue and Wildlife

Prevalence of bluetongue in wildlife has not been studied systematically due to several reasons including difficulty in collection of samples and monitoring of wild animals in their natural habitats. In addition, poor understandings of biology, immune system and behaviour of wild animals have been the reasons for scanty reports on distribution of bluetongue in free-ranging animals. However, in spite of the constraints, some studies have been conducted in Africa and North America. Based on these studies, several wild animal species were reported to exhibit a spectrum of disease manifestations ranging from inapparent to highly acute, leading to even death in certain circumstances. In addition to morbidity and mortality, BTV may induce debilitating effects, reproductive disorders, or behavioral changes in susceptible wild animal species, which in turn may substantially increase their susceptibility to other ecological mortality including factors such as predation and parasitism (Hoff and Trainer 1978). The prevalence of BTV in free-ranging wild animal population can be related or unrelated to the host density or the species specificity of the virus and its mode of transmission. In addition to being primary target of the viral infection, a number of wildlife species may also act as reservoirs, or be involved in the maintenance of natural cycle of the virus that affect domestic animals. The relationship between viral infection of wildlife and those of domestic animals has been put into two categories: (i) the aetiologic agents that produce overt signs in both wild and domestic animal species, and (ii) those agents which have not been described to produce overt disease in wildlife species but may be transmitted to domestic livestock, where these may produce severe disease manifestations (Hoff and Trainer 1978). Though wildlife species have been reported to serve as reservoirs, it is also true that certain diseases of domestic animals may be transmitted to wildlife from livestock. A number of ecological factors have been suggested to be associated with the transmission of BTV in wild animals.

Studies on BTV and epizootic haemorrhagic disease virus (EHDV) in non-domesticated artiodactyls have been largely restricted to Africa and North America. The clinical profile of the disease has been recorded to be variable, ranging from sub clinical to severe depending on species, age and resistance status of the animals. Both, BTV and EHDV have been reported to cause mortality in some species including topi, kudu, bighorn sheep, white tailed deer, mule deer, elk, muntzak, and prong antelope (Griner 1974, Hoff

et al. 1973a, Kistner *et al*. 1976, Robinson *et al*. 1967, Stair 1968b, Wells 1962). However, BTV has been isolated from only a few wild species. Successful BTV isolation efforts from the clinically normal free ranging mountain gazelle have been reported (Barzilai *et al*. 1971). Some workers have been successful in producing experimental infection in white-tailed deer, pronghorn antelope and buffalo calves (Hoffman and Trainer 1972, Thomas and Trainer 1970a, Young 1969). Blesbok, mountain gazelle, elk, and hog deer have also been reported to suffer sub clinical infections having blood viral titres sufficient to infect *Culicoides* vectors (Barzilai and Tadmor 1972, Murray and Trainer 1970, Neitz 1933, 1966). An incomplete list of wild animal species reported to be serologically positive is presented in Table 18.

A few studies have been conducted in North America in captive situation to gain clear insight into mechanism of pathogenesis of BTV and its circulation in wild animals. It was demonstrated that white-tailed deer experimentally infected with BTV exhibit almost similar gross lesions and signs as in other susceptible ruminants. The gross lesions are often characterised by extensive haemorrhages and that is why the disease is occasionally referred to as haemorrhagic disease in white-tailed deer. The death, reported in BT and EHD, is believed to be due to loss of vascular integrity and consequent disseminated intravascular coagulation. The possibility that BTV or EHDV are responsible for haemorrhagic disease in California black-tailed deer has been suggested (Jessup *et al*. 1984, 1990). However, exact diagnosis has been difficult due to lack of success in isolation of BTV or EHDV from the dead or dying deers.

Both BTV and EHDV induce similar lesions, hence it is difficult to arrive at definitive diagnosis based on clinical profile. However, now with the availability of more refined and specific serological and nucleic acid based assays.

Earlier, BTV was thought to be primarily confined to domestic and wild ruminants. However, observations made during the past one decade have clearly suggested, susceptibility of a number of non-ruminant domestic and wild animal species to BTV. Serological survey conducted in different African wildlife reserves including Moremi of Botswana, Masai Mara of Kenya, Kruger National Park of South Africa, Serengeti National Park of Tanzania, Chipangali Breeding Centre of Zimbabwe, has indicated possibility of natural infection of BTV in wild carnivores including lions, cheetah, hyena, African wild dog and others (Alexander *et al*. 1994).

Prevalence of bluetongue virus infection in artiodactyl species

The serologic evidence of BTV infection among free-ranging artiodactyl populations including bighorn sheep, barbary sheep, elk, moose, and pronghorn antelope has been reported in North America. The impact of prevalence of BTV in free-ranging wildlife populations is not clearly understood. However, recent data suggested that BT is an important ecological factor to be considered in wildlife management strategies. It has

Table 18. Different species of wildlife serologically positive for bluetongue virus infection

Common name	Zoological name	Continent where recorded
Gazelle	*Gazella gazella, G. grantii*, *G.* thomsonii	Africa
Eland	*Taurotragus oryx*	Africa
Reedbuck	*Redunca fulvorufula*	Africa
Sitatunga	*Tragelpahus spekei*	Africa
Hartebeeste	*Alcelaphus buselaphus*	Africa
Wildebeast	*Connochaetes taurinus*	Africa
Impala	*Aepyceros melampus*	Africa
Waterbuck	*Kobus ellipsiprymnus*	Africa
Orix	*Oryx beisa*, *Orix gazella*	Africa
Oribi	*Ourebia ourebia*	Africa
Bontebok	*Damaliscus dorcas*	Africa
African elephant	*Loxodonta africana*	Africa
Asian elephant	*Elephus maximus*	Asia
Buffalo	*Syncerus caffer caffer*	Africa and Asia
Camel	*Camelus dromedarious*	Africa and Asia
Yak	*Poephagus grunniens*	Asia
Reindeer	*Rangifer tarandus*	North Ameria
Mule dear	–	North America
Fallow deer	*Dama dama*	North America
White tailed deer	*Odocoileus virgianus*	North America
Black tailed deer	*Odocoileus hemioneus columbianus*	
Elk	*Cervus canadensis*	North America
Pronghorn antelope	*Artilocarpa americana*	North Ameria
Moose	*Alas americana*	North America
Bison	*Bison bison*	North America
Barbary sheep	*Ammatragus lervia*	North America
Bighorn sheep	*Ovis canadensis*	North America
Lion	*Panthera leo*	Africa
Cheetah	*Acenonyx jubatus*	Africa
African wilddog	*Lycaon pictus*	Africa
Hyena	*Crocuta crocuta*	Africa
Leopard	*Felis pardus*	Africa
Panther	*Leo panthera*	Africa
Spotted deer	*Axis axis*	Asia
Sambar	*Cervus unicolor*	Asia
Topi	*Damaliscus korrigum*	Africa
Kudu	*Tragelaphus strepceros*	Africa
Muntjak	*Muntiacus reevesi*	Africa
Kongoni	*Alcelaphus burlaphus*	Africa
Blackbuck	*Antelope cervicapra*	Asia
Blesbok	*Damaliscus albinfrons*	North America
Hunting dog	*Licaon pictus*	Africa
Striped field mouse	*Rhabdomys pumilio*	Africa
Water rat	*Odomys irroradus*	Africa
Shrew	*Crocidura* sp.	Africa

been hypothesized that BTV was the major factor in disappearance of bighorn sheep from west Texas; since bighorn sheep populations declined appreciably at the time BTV was a rampant disease among domestic sheep on the same range (Robinson *et al*. 1967). Bighorn sheep were highly susceptible to the disease in producing both morbidity and mortality (Robinson *et al*. 1967). So much so that BTV has hampered efforts aimed at restocking bighorn sheep on their former ranges. In 1976, thousands of pronghorn antelope and mule deer died due to BTV in Wyoming, Colorado, Nebraska and New Mexico. Though BT does not appear to cause spectacular epizootics in wild deers, the field data indicate that BTV is an important mortality factor of unknown magnitude among white tailed deer and mule deer fawns. In south Texas, where BTV is enzootic, antibody prevalence was as high as 89% in adult white tailed deer (Hoff and Trainer 1974a, Marburger *et al*. 1970). This indicated a very high prevalence of BTV in white-tailed deer in Texas. Additional losses were suggested to occur *in utero* when BTV transmission and the deer's gestation period overlap. Experimental infection of BTV during first trimester of gestation in white-tailed deer resulted in early absorption or uncomplicated abortions (Thomas and Trainer 1970b). These observations clearly suggested that BTV represents a potential threat to the white-tailed deer populations.

Perusal of literature on history of BT in Africa indicated that it was a viral disease of wildlife initially which was able to exploit the virgin, susceptible population of European livestock introduced to this continent. Contrary to Africa, the available data suggested that the opposite may be true in North America. Though viraemic titres in wildlife are sufficient to infect *Culicoides* vectors and thereby maintain an epizootic, all evidences suggest transmission of BTV from domestic livestock to wildlife in North America. Decimation of bighorn sheep population in west Texas is best example that linked transmission of BTV from domestic sheep to bighorn sheep (Robinson *et al*. 1967). Similar evidence for the mortality due to BTV among mule deer, pronghorn antelope and bighorn sheep following an outbreak of BT of cattle and sheep was reported from Oregon and California (Kistner *et al*. 1976). Occurrence of BTV in zoo animals after an outbreak on neighbouring sheep ranch in California has also been reported (Hoff *et al*. 1973). A similar situation occurred in Texas, where captive white-tailed deer developed BTV after infected sheep were introduced to the area (Stair 1968a). North American observations strongly suggested that outbreak of clinical BT in domestic livestock has often resulted in significant mortality in wild animals. These observations confirm the assumption that BTV is transmitted from domestic livestock to wildlife in North America. Though Asian continent has very vast and diverse wildlife, the information on prevalence of BTV is almost non-existent. The habitat and the socio-economic conditions are totally different in Asia as compared to North America. Growing human population has led to unprecedented pressure on natural habitats and man. The livestock is competing for the same habitat. This has created very favourable conditions for free flow of pathogens from

livestock to free-ranging wildlife. A limited study was conducted at Sariska Tiger Reserve, Rajasthan, India where cattle, buffalo, goat, sambar and chital sera were tested for BTV antibodies. The survey indicated presence of BTV antibodies in both domestic and wild ruminants (Prasad *et al.* 1998). Nearly 20,000 domestic livestock was dependent on the tiger reserve for feed/fodder. This suggested that the domestic animals and wildlife were using the same ecosystem for feed and water, thereby increasing the possibility of bi-directional transmission of pathogens. There is no report on isolation of BTV from wild animals in India.

Collection of captive native and exotic wildlife ruminants for exhibition, scientific study or commercial purposes are at potential risks of infection with BTV or EHDV (Hoff and Hoff 1976). Mortality and morbidity due to BTV and EHDV have been reported from zoos in several countries and herds maintained for stocking or other scientific purposes. Most of these outbreaks resulted from viral spill-over during epizootics among neighbouring domestic sheep or deer. BTV has been incriminated in majority of such episodes. Economic considerations and the fact that many animals may represent threatened or endangered species dictate the control of BTV and EHDV in zoos and other captive situations. Both the infections may be controlled by reduction of *Culicoides* populations or by immunising susceptible host populations with efficacious vaccine. In North America, immunisation of bighorn sheep and white-tailed deer has been successful (Hoff and Trainer 1974 a, and b, Robinson *et al.* 1974), however, these results might not translate to other susceptible species. The control of BTV is very complicated in free ranging wildlife. Operations in North America involving such native species as bison, moose, mule deer, elk and white-tailed deer must consider EHDV and BTV as potential threats especially in deer. With the availability of suitable vaccines and methods of administration, these risks can be minimised. Trade in animals from enzootic regions carries risk of spreading the virus to areas free of the disease. Bisons from a ranch in Florida have been refused entry into Canada because antibodies to BTV were detected in the animals (Hourrigan and Klingsporn 1975a). Similarly, New Zealand refused entry of an Indian elephant found positive for BTV antibodies. Similar restrictions have been imposed by several other countries, which are free from BTV.

Bluetongue virus infection in nonartiodactyl species

The focus of investigations on BTV in wildlife has been on the artiodactyl species. However, recent findings suggested that non-ruminant species of wildlife might have significant role in maintenance of BTV in natural habitat. Therefore, the role of nonartiodactyls in the natural history of BTV warrants systematic investigations. BTV, for example, has been repeatedly isolated from African rodents and insectivores. These include striped field mouse (*Rhabdomys pumilio*), water rat (*Odomys irroradus*) and a shrew (*Crocidura* sp) (DuToit 1955, Kemp *et al.* 1974, Lee *et al.* 1974). However, similar results have not been reported from other parts of the

world including North America where several species of wild rodents were tested but found negative for BTV antibodies. The possibility of BTV infection has been described in a variety of carnivore species. The prevalence of BTV antibodies in carnivores has been hypothesised to be because of infection of these animals from ingestion of BTV infected meat from BTV susceptible domestic and wild ruminants which are pray of wild carnivores. Domestic dogs and cats have also been found to have BTV antibodies in some African countries. The infection of dogs and cats has been ascribed to eating of meat of sheep, cattle, goats and wild bovids infected with BTV. However, if arthropod vectors such as *Culicoides* midges were responsible for transmission of BTV to these carnivores, more even distribution of BTV antibodies would have been expected (Alexander *et al.* 1994).

There are two issues which are important to understand epizootiology of BTV. The first is related to the potential impact of BTV infection on susceptible carnivore hosts and importance of this infection in carnivore population structure and regulation. BTV has been shown to cause morbidity and mortality among domestic dogs. Therefore, BTV infection may have analogous consequences for wild relatives. Second question pertains to role of carnivores in epizootiology of BTV. A more detailed study should be performed to investigate whether BTV can be transmitted from carnivores to ruminants by *Culicoides* midges or carnivores are dead end hosts that acquire infection after ingestion of BTV contaminated meat. If BTV is transmitted from carnivores by insect, what is the species of the vector that is capable of transmission of BTV from carnivore to ruminants. These issues need to be probed thoroughly to understand dynamics of BTV circulation in domestic and wild animals.

Interface conflict

Most of the wildlife reserves throughout the world are surrounded by human settlements with the considerable domestic livestock population. This situation is more apparent in underdeveloped and developing countries of Africa, Asia and Latin America where natural habitats are facing tremendous pressure from the growing human needs. This has led to the more frequent meeting of wild animals with the domestic livestock. In many wildlife reserves, a very large number of domestic livestock come for grazing. Thus share the same habitat for fodder and water. In the situations where domestic animals are competing for food and water with their wild counterparts, the possibility of the bi-directional transmission of infectious diseases from domestic to wild and *vice-versa* increase many folds. Therefore, clear understanding of the dynamics of circulation of pathogens in wildlife and domestic animals in the areas surrounding wildlife reserves and national parks would provide clues for better management of livestock and conservation of fast diminishing wild fauna.

Many facets of BTV epizootiology among wild animals remain to be investigated. Susceptibility studies need to be conducted on more species and these must include viraemia determinations. Ideally, assays for viraemia

should employ *Culicoides* vectors, since these are more sensitive in detecting virus than direct assay of the blood (Luedke *et al*. 1977). Such information would be valuable for formulating vaccination requirements of captive situations and determining whether carrier status develops. Further, this knowledge would affect wildlife management practices, such as multiple use policy of game ranges, restocking programmes, introduction of exotic wild species. Coupled with the susceptibility studies, is the need for expanded serologic surveys to refine data on host and geographic ranges of the virus on a world-wide basis. Also long-term studies are required to measure the effect of viral infections on herd recruitment and as a limiting factor of herd size.

Effective vaccines need to be developed for use on susceptible species. Since injectable vaccines are not suitable for free ranging wild animals, efforts should be made to develop oral vaccines and development of methodology for distribution of vaccine to free-ranging animals. The natural history of BTV in enzootic situations needs elucidation with regard to mechanism of inter-outbreak maintenance of the virus. All modes of transmission should be considered. The role of non-ruminant species requires considerable research efforts. Over wintering mechanism for the viruses also need to be worked out. Cattle are capable of serving as an over wintering host for BTV (Houringan Klingsporn 1975b) and it has been suggested that *Culicoides* may also serve in this capacity. Other over wintering mechanisms for virus are a matter of conjecture. Perhaps the non-ruminant vertebrate host may be serving as over wintering host.

Entomological investigations are needed to determine the vector capacity of various *Culicoides* species, for BTV and EHDV. However, only limited data are currently available on these insect vectors. Such data must be coupled with field studies on the host feeding preferences to allow better understanding of epizootiology of these virus diseases as well as possibly permitting the development of biologic control measures using resistant *Culicoides* strains. There is total lack of information on the status of BTV infection in wildlife in India. The understanding of prevalence of BTV in free-ranging wild animals would help in understanding the BTV infection in domestic animals. Therefore, comprehensive studies involving wildlife reserves vis-à-vis livestock should be conducted to gain clear understanding on dynamics of BTV circulation in India.

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6

Vector

Mosquitoes were for the first time implicated in the transmission of viral diseases more than a century ago (Reed 1902). Since then, a large number of viral diseases of man and animals have been found to be transmitted by a variety of insect vectors. Vector-borne viral diseases have been causing severe economic losses to animal husbandry sector. About 500 animal viruses are now known to be transmitted by insects. These viruses are collectively called arboviruses. Advances in molecular biology in the past over three decades have enabled investigators to genetically modify viruses and insect vectors. This has resulted in spurt of research endeavours aimed at understanding the molecular and genetic basis for transmission of viruses by the vectors. The data generated in the past 10 years has provided very interesting insight into the biology of the vectors, their competence to transmit viruses to vertebrate hosts (Gray and Banerjee 1999).

Amongst hundreds of vectors, *Culicoides* midges have great economic significance as transmitters of animal diseases. *Culicoides*, also known as biting midges, sand flies, biting gnats or *no see ums* or pinky midges have enormous importance to medical and veterinary entomology. They belong to the family *Ceratopogonidae* and order Diptera. More than 1,400 species of *Culicoides* have been identified all over the world. Of these, more than 90% are obligate blood suckers i.e. haematophagus (Mellor 1996). *Culicoides* occur in all inhabited continents of the world. However, there are no reports of prevalence of *Culicoides* midges from New Zeeland and Patogonia. They are the smallest blood sucking insects which feed on warm blooded vertebrates. They transmit the important animal viruses, viz. BTV in domestic and wild ruminants, African horse sickness virus (AHSV) in equines, epizootic haemorrhagic disease (EHDV) and Akabane viruses (AV) in wild ruminants such as deer (Mellor 1996). A few species of the vector cause severe dermatitis in horses. However, this chapter is intended to provide current understanding of *Culicoides* midges in relation to vector biology, vector capacity and competence and transmission of BTV, which is prototype of *Orbivirus* genus.

Morphology and identification of adult *Culicoides*

The size of adult *Culicoides* varies from 1-3 mm in length with light and dark markings on the wings (Mellor 1996). At rest, wings are held vertically over abdomen. Antennae are 15 segmented and are plumose in

males while pilose in females. Mouth parts of females form a beak or rostrum which is used for sucking blood. A variety of characters have been used to identify different species of *Culicoides*. Main features of head, thorax and abdomen have been the basis for identification (Fig. 12). Following criteria are generally used for taxonomical characterisation of *Culicoides* midges:

Female antennae: The antenna has 15 divisions (segments). The two nearest the head (scape and pedicel) are not important, but the flagellar segments (segment 3-15) may bear a number of small sensory pits (sensillae). The number, shape and distribution of these is of great importance in classification and many keys are based on them. However, before they can be examined clearly it is necessary to slide mount individual midges. Once mounted, midges may be examined under a × 200 to × 400 magnification.

Male genitalia: In male midges the plumed antennae are not of much use in identification but prominent genitalia are of great importance. Several features of these are of significance. In particular, the proximal segment (basistyle) of the 2 segmented claspers has 2 internal processes at the base, a ventral root and a dorsal root. The shape and size of these are important. The aedeagus which is basically, a highly variable Y shaped structure which forms a support for genital duct. All of these structures vary in size and shape dependent on species.

Wings: The wings of *Culicoides* bear dense microtrichiae and it is the size and pigmentation of these that give rise to the characteristic patterns of dark and light spots in most species. These patterns and their positions in relation to various veins and cells are of primary importance in classification and provide probably the best set of specific characters. It is also significant that most of the wing characters can be distinguished without slide mounting and under × 4 to × 20 magnifications. It is not possible to distinguish all species of *Culicoides* by wing markings alone, since closely related species may have vertually identical markings, and a few species have no wing markings at all. However, for most purposes, other than those of the specialised taxonomist they are sufficient, and numerous keys have been based on them. Some of these keys are very complex and require some experience to use them effectively in the identification of *Culicoides* species.

Life-cycle of *Culicoides*

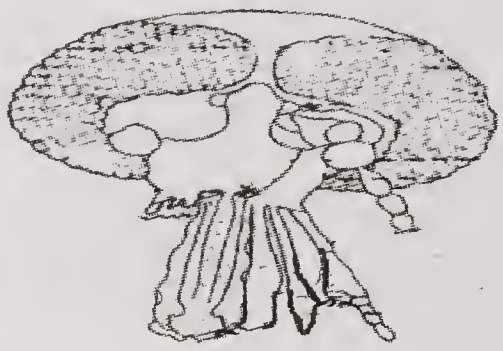
Usually females are haematophagous and the blood-meal is used as rich source of proteins for development of eggs. Shallow pools, stagnant waters, pipe leaks, spillage from pens, marshy areas and dung heaps offer good breeding sites. Some species may breed in muddy pasture lands, sugarcane and banana fields or in dung pits, moist soil or at the margins of dung fouled water. Eggs are laid on stagnant water surface, banks of ponds, damp soil, dung heaps etc. in batches with up to 30 eggs/batch. Most species of *Culicoides* are crepuscular, that is the adults are active particularly around sunset and sunrise, and to a lesser degree through night. However, the timing of this activity is controled mainly by the level of light intensity, and

Thorax with halters

Leg from base to claw-end

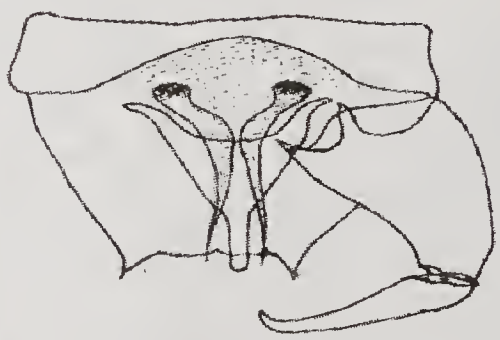
Antenna showing antennomeres

Wing showing veins and cells



Dorsal view of head and mouth

Antennae showing spines and hairs



Ventral view of male genitalia

Maxillary palp

Tip of hind tibia with spine and comb

Fig 12. Diagrammatic representation of different parts of *Culicoides oxystoma*.

so on days, the biting of crepuscular species may be extended throughout the day. Flight activity of *Culicoides* is undertaken for 3 reasons, viz. mating, seeking a blood meal and seeking a oviposition site. Wind speed above 5 kmph tends to curtail flying and in general the actual distances flown by *Culicoides* are very short. Generally the *Culicoides* powered flight distances are less than 5 km in 24 hr. However, occasionally midges may be conveyed on wind, as aereal plankton, over much greater distances than this. In the South China Sea, live *Culicoides* have been collected in ship over 161 km out to sea. This sort of distance has considerable implications in regard to disease transmission and there is a considerable body of circumstantial evidence to suggest that various outbreaks of BTV, which occurred in areas several hundred kilometres from the source of infection, may have been initiated in this way.

Mating typically takes place in flight. The males form swarms and the females fly through the swarms and are captured by males. During swarming, male *Culicoides* bring the setae of the antennal plumes erect, in which position they can recognise the wing-beat frequency of the females. When swarming, males always orient up-wind and they use a variety of markers to stabilise the position of the swarm. In *Culicoides* species both sexes are attracted to host and the males mate with the females shortly after the latter has taken the blood meal. A few *Culicoides* species are able to mate with swarming. In these cases both sexes run about on the soil or vegetation, usually near breeding site and the males mate on contact with female. Most species of *Culicoides* mate only once in life time although some have been observed to mate several times. The sperms are passed to females in a spermatophore and are stored in female's body in spermathaecae. The eggs are about 0.25 mm long and are banana shaped. When laid they are white, but darken rapidly to brown or black. Unlike some mosquito eggs they can stand prolonged drying and usually hatch within few days. With haematophagous females, a blood meal is usually required for each egg batch and in *C. variipennis,* for example, a single female may live 44 days and deposite up to 1,000 eggs in as many as 7 egg batches during her life time.

Oviposition occurs mostly in afternoon whereas swarming and mating occurs before sun set. Eggs hatch in about three days. All species of *Culicoides* exhibit complete metamorphosis. The life-cycle of the midges include four stages, viz. eggs, larva, pupa and adult. Larvae are aquatic or semi-aquatic in nature and are whitish in colour. There are four larval instars (Blanton and Wirth 1979). Pupation occurs above water surface. One life-cycle is completed in 25-35 days in warm weather and is extended up to two months in colder conditions. Different stages in life-cycle and their approximate duration are presented in the Table 19.

The immature stages of *Culicoides* require some amount of free water in the environment. Therefore, depending upon species, the immature stages may be found in irrigation pipe leaks, swamps, beaches, marshes, bogs, streams, pools, tree holes, rotting vegetation and even cow pats. They are rarely or never found in large, open bodies of water such as lakes, reservoirs

Table 19. *Culicoides* life-cycle stages

Stages	Duration
I. Eggs (10-150 per oviposition)	Few days
II. Larva (4 instar)	Few days to several months
III. Pupa (almost non -motile)	Few days
IV. Adult	Few days to >1 month

and rivers, except around the edges at the land-water interphase. The life cycle of *Culicoides* may take as little as 7 days at high ambient temperature (tropics) but may extend for more than 6 months in temperate and cool countries, and in fact most *Culicoides* overwinter as fourth instar larvae. This way of overwintering is called diapause and is a physiological mechanism. It is controlled by environmental factors including temperature and specially the number of day light hours. Some species of *Culicoides* in some temperate countries do not have diapause and in these situations adults can be collected at any time of the year. *C. obsoletus* is one of the examples of this type of midge.

Concentrated sucrose or 30% magnesium sulphate can be effectively used to collect larvae and pupae of most species of *Culicoides* from substrate using floating out procedure. Once in liquid phase the pupae tend to float since they are almost non-motile but the larvae of most species swim with characteristic serpentile motion. However, the *Culicoides* species, which inhabit fairly dry or dense habitats such as wet soil or animal dung, floating out is not effective. In these cases larvae (but not pupae) may be collected by use of Berlase funnel. In whatever type of habitat they may be found, the larvae and pupae tend to inhabit the 2.5 or 5 cm of the substrate.

Since immature stages of most of the *Culicoides* species have not been described yet, the collection of larvae and pupae have limited value. Therefore, in view of the difficulties in identification of immature stages, most researchers either collect live larvae and pupae, which they attempt to rear to adult, or else concentrate on collection of adults alone. A number of ways including sticky traps, bait animals, truck traps, suction traps, emergence traps, CO_2 traps and light traps (Fig. 13) have been devised to collect adult *Culicoides* specimens for identification, virus isolation and other types of studies on vector biology.

Vector capacity and competence

All the *Culicoides* species which feed on vertebrate hosts are not capable of being infected with viruses or transmitting the viruses. Traits associated with vector ability to transmit pathogens, such as host preference, biting or feeding rates, gonotropic cycle, population densities and vector longevity, determine vector capacity (Nunamaker *et al.* 1997). The vector capacity is also dependent on vector competence. The species, which are susceptible to oral infection, may not be able to transmit the virus orally. A multiple barrier system appears to be associated with the virus infection and multiplication

Fig 13. Battery operated light trap used by the authors for trapping *Culicoides* midges.

(Fig. 14). The mechanisms controlling the oral infection operate at the level of midgut wall i.e., mesenteron infection barrier (MIB) and mesenteron escape barrier (MEB). MIB, the virus is unable to penetrate from gut lumen into the mesenteron. It has been observed that if mesenteron is punctured, infection is initiated. MEB operates when the virus is unable to disseminate from the infected gut cells to the haemocoel by dissemination barrier (DB). Similarly, salivary gland infection barrier (SGIB) and salivary gland escape

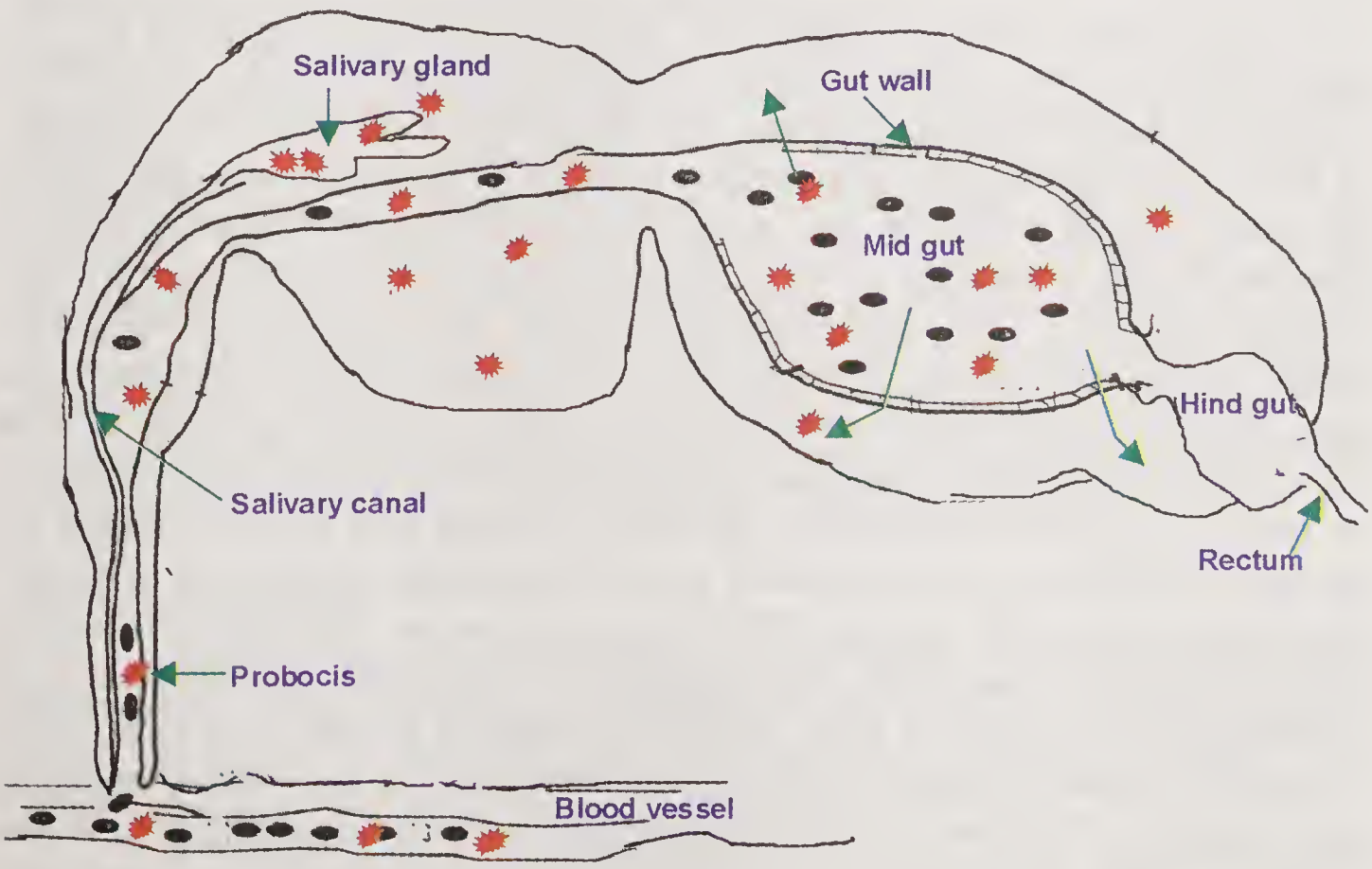


Fig. 14. Virus transmission steps involved after taking of blood meal by *Culicoides*.

barrier (SGEB) also operate which may prevent the virus transmission (Fu *et al*. 1999). The absence of evidence for transovarian transmission of BTV strongly indicates presence of transovarian transmission barrier (TOTB). Keeping in view the above barrier systems, susceptibility of a species to infection along with proportion of population, which is actually able to transmit the virus could account for the vector potential.

According to a study, the vector containing 2.7-5.1 $\log_{10}TCID_{50}$ virus/fly, regularly transmitted the virus infection to the susceptible host while midge with less than 2.5 $\log_{10}$ $TCID_{50}$ virus/fly failed to transmit the infection (Jennings and Mellor 1987). The study further suggested that more *C. variipennis sonorensis* were infected if fed on blood meal containing 10^6 pfu/ml than those fed on lower concentration and no flies were infected from blood meal containing 10^4 pfu/ml (Jones and Foster 1971). This indicated that level of viraemia also determines vector capacity. It has also been observed that nutritional status of larvae also influence susceptibility to the viral infection. Poor larval nutrition and crowding resulted smaller *C. variipennis sonorensis* females which were more susceptible as compared to larger females (Tabachnick 1996).

The genetic makeup of a species affects its efficiency as a BTV vector. Recent studies have suggested that the most important barriers to BTV transmission in *Culicoides* vector species appeared to be a mesenteron infection barrier, which controls initial establishment of persistent infection, a mesenteron escape barrier which can restrict the virus to gut cells and dissemination barrier, which can prevent virus that enters haemocoel from infecting secondary target organs (Fu *et al*. 1999). *Culicoides variipennis* do not appear to present either SGIB, or a SGEB to BTV (Fu *et al*. 1999).

It has been observed that the virus distribution is limited to the geographical areas, which contain competent vector species. A positive correlation has been made between the infection in *Culicoides* and incidence of virus in vertebrate host. The outbreaks of BTV have been associated with the peak activity period of the vector. In other words the activity peak is associated with seasonality of BTV. During unfavourable conditions for the midge, when its activity becomes very less, the virus may be maintained in cattle, buffalo, sheep and other ruminants. It has been reported that the virus may circulate in sheep for about 31 days while in bovine as long as 700 days (Tabachnick 1996). However, the viraemia sufficient to cause infection to vector is generally lesser than 30 days in sheep and several months in bovines. *Culicoides* may also survive as over wintering midges and maintain the virus during this period. The virus can persist through-out winter provided that the length of cold weather is less than the survival period of *Culicoides*. If the winter is long, the over-wintering of the virus may occur in ruminant host. The mechanism of over-wintering has not been adequately characterised. Therefore, possibility of vertical transmission of BTV viruses in the *Culicoides* vector must be considered as an unidentified over wintering non-ruminant vertebrate host (Barratt-Boyes 1995). The infectivity period

of the vector may be longer at lower temperature. Spread of the virus occurs through the flight of infected midges or they may be carried on the wind to non-infected areas (Tabachnick 1996).

Genetic basis of vector competence

Focus of research, in the past few years, has been on determining the genetic basis of vector competence. The preliminary studies have indicated that the susceptibility to the viral infection is governed by single gene locus. However, more studies are required to fully understand the genetic mechanism controlling vector capacity and vector competence (Tabachnick 1996).

Molecular basis for virus-vector interactions that regulate transmission are not well understood, but it is clear that genetic elements within both virus and the vector ultimately decide if a particular species or individual within a species of arthropod is able to be a vector for a particular virus strain. Environment or abiotic factors also play a role in determining virus-vector interactions. The genetics of vector competence is receiving widespread attention and the result obtained so far have begun to change the central dogma that all individuals within a vector species are potential vectors. A more enlightened concept states that populations within a species will differ in their ability to be efficient vectors for certain viruses (Tabachnick 1994). Understanding why the vector is a vector and developing the tools to rapidly and accurately identify potential vectors is important for understanding epidemiology of BTV and for developing control measures. Currently viral disease control programs are aimed at protecting the host using vaccines. However, the more direct approach would be to prevent the infection of arthropod host and/or transmission of the virus. Arbovirologists and the entomologists have begun to develop systems to investigate the genetics of vector populations and develop tools to allow the molecular mapping of elements that differ between individuals that are allele to efficiently transmit viruses and individuals that are refractive to virus infection or transmission (Tabachnick 1994). In contrast to simple monogenic control of vector susceptibility to malaria parasite or filarial infection, the competence of viral vectors appears to be complex multigenic phenomenon in several instances (Tabachnick 1994, Tardieux *et al.* 1991). However, a single locus that controls the susceptibility of *Culicoides variipennis* to BTV virus was identified (Tabachnick 1991).

Virus competence

It is odd, that the arboviruses are classified as vertebrate infecting rather than being classified as invertebrate viruses. The arthropod host is more important in the evolution and survival of the virus because it will exert a greater selection pressure (Nuttal *et al.* 1991).

The competence of virus transmission by vector is not controlled solely by the vector; the virus also contributes to the overall process. The limited information is available on the genes and gene products of viral origin that

influence vector transmission. The availability of continuous cell lines of vector origin has greatly enhanced our understanding on orbivirus-vector interaction. Insect cell lines facilitated investigations on virus entry into and release from insect cells and of virus replication and gene expression in insect cells relative to their animal host counter parts (Miller and Brown 1991). Xu *et al.* (1997) hypothesised that after ingestion of BTV particles from the infected blood into the vector gut, outer capsid proteins VP2 and VP5 of the virus are partially degraded and VP7 is exposed to midgut peritropic membrane. The VP7 protein of the virus would then initiate specific binding of the virus to the membrane receptors and the partially degraded VP2 and VP5 may facilitate entry of the virus into gut cells. The experimental evidence shows that VP2 helps in penetration of the virus (Brookes *et al*. 1994). Xu *et al.* (1997) suggested that VP7 protein plays a crucial role in initiation of BTV infection in insect vector and evidence for the potential receptor protein (23 kDa) in the insect vector. Further investigations on these proteins would provide insight into mechanistic interaction of these proteins with VP7 and the potential importance of these proteins in vector competence.

Transmission of bluetongue virus

In 1944, *C. imicola* was for the first time linked with transmission of BT disease in sheep in South Africa (Du Toit 1944). Subsequently, it was demonstrated that *C. imicola* was a major vector of BTV in Africa, Middle East, Israel, Vietnam, Turkey, Iran, Cyprus, Greece, Portugal and Spain while *C. variipennis* was predominant vector in USA and Canada (Mellor 1990). *C. oxystoma* is widely found across South East-Asia including India. In northern India, *C. oxystoma* was identified as a potential vector of BTV (Bhatnagar *et al*. 1997). So far 17 species of *Culicoides* have been associated with BTV and only eight of these have been proved to transmit BT viruses to vertebrate host. As it is apparent from the Table 20-21, there is a great diversity of *Culicoides* midges which are endowed with the ability to transmit BTV virus to susceptible host in different regions of the world (Bhatnagar *et al*. 1995). This diversity appears to be due to vector capacity and competence which are influenced by genetic and geoclimatic factors.

Culicoides females seeking a blood meal are attracted to a host by its size, shape, odour, colour and exhalations. Carbon dioxide (CO_2) is a very important attractant (Nelson 1965). On biting an infected host, the female ingests infected blood containing virus. The virus penetrates gut wall of the midge and multiplies in the tissues (Sellers 1980). After infection of the cells of mid gut, replication of the virus occurs and progeny virus is released into the haemocoel. Infection passes on to the secondary target cells particularly fat bodies and salivary glands (Chandler *et al*. 1985, Bowne and Jones 1966). While taking a blood meal, the virus passes on to the healthy host and the disease cycle is maintained.

When in abundance, the biting rate of midges may exceed 20 bites/ cm^2 of the exposed skin/minute (Mellor 1996). Approximately 10 μl blood

Table 20. *Culicoides* species recorded from different parts of the world in relation to BTV transmission

Vector species	Region/country
Culicoides brevitarsis	Australia, South-East Asia
Culicoides actoni	Australia, East Asia, South East Asia
Culicoides imicola	Africa, Middle East, Mediterranean countries,South Asia, East Asia
Culicoides insignis	South America, North America, Central America
Culicoides obsoletus	Bulgaria, Mediterranean countries
Culicoides pusillus	Central America, North America, South America
Culicoides variipennis sonorensis	Central America, North America, South America
Culicoides wadai	Australia, South-East Asia
Culicoides bolitinos	South Africa
Culicoides fulvus	Australia, East Asia, South-East Asia
Culicoides variipennis	Canada, North America
Culicoides oxystoma	China, India
Culicoides homotomus	China
Culicoides cornutus	South Africa
Culicoides brevipalpis	Asia, Australia
Culicoides filariferus	Caribbean, Central America
Culicoides boydi	California, USA

is ingested by the females of *C. nubeculosus* and *C. variipennis*. This viraemic blood contains 10^5 to 10^6 $TCID_{50}$ of the virus/ml (Mellor and Boorman 1980, Mellor 1990). Hence each midge will ingest about 10^3 to 10^4 $TCID_{50}$ of the virus (day zero value). After ingestion of the viraemic blood, the virus becomes undetectable for some time in the infected midge (Foster and Jones 1979). This stage is generally called as eclipse phase. The initial decrease in the virus titre or the undetectability may be due to digestion of infected blood meal with attachment, penetration and uncoating of the virus in midgut cells of infected midges. Subsequently, replication of the virus occurs in susceptible tissues of the midge (7-9 days post infection) and concentration of the virus increases to 10^3 to 10^4 fold (10^6 to 10^7 $TCID_{50}$ of the virus/midge). This level of virus is maintained in the midge. The static stage achieved may be due to the cessation of virus multiplication with retention of infectivity or a steady state of virus replication with its corresponding inactivation. Overall controlling factor may be the number of susceptible cells available within each infected midge. Further transmission to a vertebrate host becomes possible at 10-14 days post infection. Viraemia sufficient to cause infection to vector insect is generally lesser than 30 days in sheep. A single infected midge can transfer virus from animal to animal and a single bite is sufficient to infect a susceptible animal.

Currently there is evidence that saliva components contribute, negatively or positively, to the transmission of arboviruses by the

Table 21. Different species of *Culicoides* reported from India

Species	Indian record
Culicoides actoni (Smith)	West Bengal, Bihar
C. anophelis (Edwards)	West Bengal, Assam, Bihar
C. arakawae (Arakawa)	West Bengal
C. autumnalis (Sen and Dasgupta)	West Bengal
C. brevitarsis (Kieffer)	West Bengal
C. candidus (Sen and Dasgupta)	West Bengal
C. certus (Dasgupta)	West Bengal
C. circumscriptus (Kieffer)	West Bengal, Bihar and Orrisa
C. clavipalpis (Mukerji)	West Bengal, Bihar
C. definitus (Sen and Dasgupta)	West Bengal
C. distinctus (Sen and Dasgupta)	West Bengal
C. drydaeus (Wirth and Hubert)	West Bengal
C. dumdumi (Sen and Dasgupta)	West Bengal
C. flaviscutatus (With and Hubert)	Maharashtra, West Bengal and Assam
C. fortis (Sen and Dasgupta)	West Bengal
C. fulvus (Sen and Dasgupta)	West Bengal, Tamil Nadu
C. hegneri (Causey)	West Bengal
C. homotomus (Kieffer)	West Bengal
C. huffi (Causey)	West Bengal, Tamil Nadu
C. imicola (Kieffer)	West Bengal
C. indianus (Macfie)	Karnataka
C. inesploratus (Sen and Dasgupta)	West Bengal
C. innoxius (Sen and Dasgupta)	West Bengal, Bihar
C. inornatithorax (Dasgupta)	West Bengal
C. insolens (Chaudhuri and Dasgupta)	West Bengal
C. kamrupi (Sen and Dasgupta)	Assam
C. macfiei (Causey)	West Bengal
C. majorinus (Chu)	West Bengal
C. orientalis (Macfie)	West Bengal
C. oxystoma (Kieffer)	Assam, West Bengal, Bihar, Rajasthan, Tamil Nadu, Punjab, Haryana, Himachal Pradesh
C. palpifer (Dasgupta & Ghosh)	West Bengal, Bihar
C. paraliui (Dasgupta)	West Bengal
C. parararipalpis (Dasgupta)	West Bengal
C. peliliouensis (Tokunaga)	West Bengal
C. peregrinus (Kieffer)	Assam, Bihar, West Bengal, Andhra Pradesh, Orrisa
C. raripalpis (Smith)	West Bengal
C. rariradialis (Dasgupta)	West Bengal
C. rarus (Dasgupta)	West Bengal
C. shortti	Assam, West Bengal
C. sikkimmensis (Dasgupta)	West Bengal

Adapted from Dasgupta (1995).

phenomenon called saliva activated transmission (SAT) (Nuttall *et al*. 1994). SAT potentiates the transmission of some arboviruses through the release of pharmacologically active substances in saliva into blood stream of the vertebrate host. These substances have vasodialatory (Perez de Leon *et al*. 1997), antihaemostatic (Valenzuela *et al*. 1996) and host defence supression (Kubes *et al*. 1994) properties.

Shroyer (1994) suggested that predictive factors such as (i) population density of vector species, (ii) vector population dynamics and age structure, (iii) weather parameters that affect vector numbers and the host, (iv) the immunological status of the vertebrate host, (v) the prevalence of infected vectors in the field samples, and (vi) virus activity during the preceding year, play important role in transmission of BTV in susceptible vertebrate host. Although many of these factors influence likelihood of a vector becoming infected with BTV, three biological factors, viz. (i) percentage of infected hosts in the study area, (ii) percentage of the vectors that feed on viraemic hosts and subsequently become infected, and (iii) the life-span of the vector in context of the extrinsic incubation period, are critical for the vector infection.

Nunamaker *et al*. (1997) have proposed a model to understand ecology of BTV-vector interaction. According to this model three very important factors are: the percentage of viraemic ruminant hosts in the study area; blood fed *Culicoides* that become infected following a viraemic blood meal; and daily survivorship data of *Culicoides*. Previous studies have suggested that 3-10 % of sheep and cattle are likely to be viraemic in a particular time and space in an endemic area (Barber and Jochim 1975, Foster *et al*. 1980) whereas 10-30% *Culicoides* become infected after taking viraemic blood meal (Jones and Foster 1974, Jones and Foster 1978 a, b). The daily survivorship of *Culicoides* ranges from 36-95% (Work *et al*. 1991). Foster *et al*. (1963) and Foster and Jones (1979) have suggested that the extrinsic incubation period for BTV in *C. variipennis* is at least 10 days. Using Nunamaker *et al*. (1997) model and the above values, number of infected *Culicoides* can be estimated in field sample of 1,800 parous females. If it is presumed that there are 3-10 % ruminant hosts viraemic in an area, then between 54 and 180 of the 1,800 midges sampled would have taken blood meal from a viraemic animal. Then if we assume that 10-30 % midges that fed actually became infected (Jones and Foster 1974, 1978 a, b), then 5-54 midges in the pool of 1,800 would be infected. Finally, when daily survivorship of 36-95 % is accounted (Work *et al*. 1991) in the context of 10 day extrinsic incubation period (Foster *et al*. 1963, Foster and Jones 1979), it is reasonable to predict that only 0.01-63% of 5-54 midges would be alive at the end of 10 days. Consequently, 0.0005-34 midges would be expected to test positive for BTV. Thus from this model one would expect only 0-34 midges to test positive from the original sample of 1,800 midges. The low rate of infection (about 0 to 1.5 %) of the *Culicoides* midges is due to a number of factors associated with ruminant host, viruses, vector biology and environment. *Culicoides* species recorded from different parts of the world

in relation to BTV transmission are given in Table 20.

Indian situation

In India, Dasgupta and his collaborators have done extensive work on *Culicoides* taxonomy, biology and ecology. Over the past 45 years, they have done pioneering work and described several new species (Table 21) of *Culicoides* midges from India (Dasgupta 1995, Dasgupta 1961, Sen and Dasgupta 1958, Sen and Dasgupta 1959). However, Dasgupta and his associates have not studied the midges with the objective of their role in transmission of diseases and their studies do not cover entire country.

Very little work has been done on characterization of *Culicoides* species responsible for transmission of BTV in India. Different species of *Culicoides* reported from India are summarized in Table 21. In 1988, BTV was isolated from *Culicoides* midges trapped from the Central Sheep Breeding Farm, Hisar, Haryana (Jain *et al.* 1988). However, species of the vector could not be identified then. Subsequently, *C. oxystoma* was identified predoimantly in the animal farms where BTV seroconversion occurred. On this basis, it was suggested that *C. oxystoma* is potential vector of BTV in India (Fig. 15). However, BTV could not be isolated from *C. oxystoma* in different isolation trials, may be due to insufficient number of the midges processed for the virus isolation in BHK 21 cells or the virus may have not been present in trapped *Culicoides* populations. The virus may pass regularly through populations of sheep/ cattle and *Culicoides* but there may be an interval of a few days between 2 successive waves of virus and the insect trapping might have coincided with these intervals. However, BTV antibodies were detected in serum samples collected from different animal farms in Punjab, Haryana, Himachal Pradesh and Rajasthan suggesting that BTV was circulating in these farms. Repeated trapping of a single species of *Culicoides* (*C. oxystoma*) from the sentinel herds/flocks, its close association with sheep or cattle and continuous seroconversion in sentinel herds established in Punjab, Haryana, Himachal Pradesh and Rajasthan strongly points towards *C. oxystoma* as potential vector of BTV in this region (Kakker *et al.* 1996, Bhatnagar *et al.* 1997). However, isolation of the virus from the trapped *C. oxystoma* and experimental transmission of the virus to the susceptible animals by it are required to confirm its vectorial role.

Fig 15. *Culicoides oxystoma* traped from northern India.

The *Culicoides* vectors can be trapped by using battery operated traps (Fig. 13) from dusk to dawn. *C. oxystoma* is more active at dusk while in areas where

day temperature remains high, adult activity may continue during night. The vector prefers calm air or slight wind with high relative humidity and temperature around 32°C for biting and multiplication. It attains a major peak in August while a minor in March. Before and after this period, population remains low due to unfavourable climatic conditions. It has been further observed that the *Culicoides* midges multiply rapidly during monsoon season (July-September) which coincides with the seasonal spread of BTV which generally occurs during or after rains or prior to winter. It has been observed that cattle are more preferred host as compared to sheep. In general upper part of host is preferred for biting but some species prefer lower part (Bhatnagar *et al*. 1995).

Culicoides are highly adapted to a wide range of temperature and moisture. In tropical areas moisture is maintained by rain water while in subtropical areas it is through irrigation water. In south India, the monsoon season (June to December) with temperature ranging from 21.2° to 35.6° C appears to be favourable period for the multiplication of the vector resulting in more outbreaks. BTV outbreaks in Karnataka, Tamil Nadu and Andhra Pradesh were associated with peak activity period of *Culicoides* spp. In Marathwada region and Kolkota, *C. schultzei* was the predominant species (Dasgupta 1995). Maximum temperature 30° to 33° C, minimum temperature 23° to 24° C; relative humidity, 80 to 85 % with low wind velocity appears to favour the multiplication of *Culicoides* vector. However, characterisation of *Culicoides* species responsible for transmission of BTV in southern India has not yet been done.

It has been observed that *C. oxystoma* multiplies rapidly during monsoon season (July-September), which coincides with the seasonal spread of BT disease in northern India. It generally occurs during or after rains or prior to winter (Bhatnagar *et al*. 1997). Detailed studies on *C. oxystoma* are still lacking as the species has been synonymized and confused in literature with other species, viz. *C. schultzei, C. alatus, C. housei, C. kiefferi, C. mesopotamiensis, C. pattoni* and *C. punctigerus. C. oxystoma* is abundantly present in India. It has been observed that about 79% is *C. oxystoma* in the total *Culicoides* population and it shows a major peak on its incidence in August and another minor peak in March in West Bengal. This species is nocturnal and more active between 7 pm- 10 pm. Sen and Dasgupta (1958) noted that 36% of wild caught females having abdomen engarged with the blood reacted with anti-cattle sera. Muddy substrate collected from margins of stock ponds around Kolkata were found to harbour the immature *C. oxystoma* indicating natural breeding sites of the species. Swarms of *C. oxystoma* are often seen in early morning close to cattle sheds. The gravid females having 115-120 mature eggs in the abdomen per natural individual of *C. oxystoma* have been caught from such swarms. The size of the eggs of this species has been recorded as 0.3 × 0.06 mm. The life-cycle of *C. oxystoma* is completed in 28-32 days in favourable environmental conditions. *C. oxystoma* has been reported to feed on cattle, preferably in the lower parts.

India has diverse ecological zones, therefore, it is likely that there are several species of *Culicoides* that have potential to transmit BTV virus. However, studies on vectorial role of *Culicoides* midges are lacking in India.

Genetic maps are being constructed for several insect vector species that are vector of important human and animal diseases. Although genetic mechanisms regulating vector competence for orbiviruses remain largely unknown, modern molecular technologies are likely to allow the identification of genetic elements in the future.

Vector control

Targeted application of known low mammalian toxicity insecticides, e.g. synthetic pyrethroids, such as with deltamethrin (weekly) or fenvalerate (every second day) in and around animal housing and directly to the target host animals should be effective. Intradermal or subcutaneous inoculation of systemic ivermectin is also effective at killing biting *Culicoides*. An additional advantage with ivermectin, and with such insecticidal food additives as tetrachlorvinphos, is that these drugs are eliminated in the faeces, which if deposited on breeding sites, are toxic to the immature stages of *Culicoides*. Ear tags impregnated with synthetic pyrethroid insecticides are effective in reducing the number of bites or strikes on cattle and sheep by a number of other insect species. These ear tags may be useful for *Culicoides* control also.

Application of larvicide abate (5% temephos granulated with gypsum) to *Culicoides* breeding sites provides a slow but sustained release of the insecticide for up to 30 days.

Vector repellent such as di-ethyl tolumide (DEET) is the only commercially available repellent with significant deterrent effect against *Culicoides* for periods of up to 4 hours. DEET applied to target animals at dusk before the peak attack period during the first 4 hours of night may have significant but temporary effect in reducing the biting rate of vectors.

Impeding danger of global warming might have serious consequences in spread of vector transmitted disease to currently disease free regions of the world. The global warming could allow movement and survival of vectors to the presently unfavourable climatic zones. Molecular studies carried out so far have suggested possibility of genetic control of vector competence. The interaction of different viruses (serotypes/topotypes/strains) with different cells of the vector species is poorly studied. Therefore, the future studies should be directed to understand differential virus-vector interactions. The clear understanding of mechanism of the vector and virus competence, host and ecological factors could provide possible approaches to biological control of vectors and develop eco-friendly technologies for management of this economically important disease. There is a complete lack of knowledge about the possible vectors of BTV in India. No systematic work on the vectors has been done so far. Considering the immense economic

significance of BTV and increasing trend of outbreaks of the disease in the past couple of years, it is urgently required to initiate an international network research programme on BTV vectors. So that potential vectors could be screened for identification, virus isolation, susceptibility to infection and the virus transmission to vertebrate host. Studies on vector behaviour, biology and population dynamics including role of biotic and abiotic factors will lead to a clear understanding about vector survival and multiplication.

Better morphological and molecular tools to identify and distinguish members of *Culicoides* complexes are urgently required. Molecular tools should be developed in such a way that these could identify haplotypes and used in monitoring and movement of vector populations. Phylogenetic analysis of nucleotide sequences of multiple genes of the *Culicoides* vectors of Europe, Asia, Africa, South America, North America and other parts of the world should be done to identify the relationships between known and novel vector species.

The international vector and the virus surveillance program will help in developing vector control strategies. Forecasting of vector-borne disease outbreaks through the development of appropriate models could be of great value in controlling BT disease. Possibility of development of genetically engineered lines of *Culicoides* midges resistant to BTV have been raised due to the preliminary results obtained in the past few years. The release of genetically modified BTV resistant vector lines in the field could gradually replace the susceptible lines. Development of sterile male lines will further help in suppressing the vector population. Further, molecular studies for understanding the mechanism of competence could provide valuable information, which may be exploited for devising the vector management strategy.

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7

Clinical Disease

A previously unknown disease, characterised by high-rise of temperature, oral lesions and inflammation of face and coronary bands, was noticed among imported European sheep by veterinarians in South Africa during 1876. The disease was recognised as a new disease of sheep and named as bluetongue. Later on detailed field investigations were conducted on this new disease and its clinical profile was described during first decade of 20th century (Hutcheon 1902, Spreull 1905). These workers were the first investigators who suggested that the new disease was non-contagious, infectious and probably vector-borne, although nature of the causative agent was not known during that time. After several years of the initial report of clinical bluetongue in South Africa, it was observed in other countries in Asia and North America. Clinical manifestations are dependent on a variety of factors related to host, vector, virus and the environment. Genetic makeup of the susceptible host is very important in determining the clinical response. BTV isolated from infected cattle often needs couple of passages in susceptible sheep before it will induce clinical disease in them. Bluetongue being mainly an inapparent infection of cattle, it is estimated that only 2% of the naturally infected cattle might be observed to have severe clinical signs of the disease (Bowne 1971). Although BTV may infect many species of domestic and wild ruminants, clinical disease is generally observed in sheep. Hence, most descriptions about clinical manifestations pertain to sheep.

Disease in sheep

All the breeds of sheep are susceptible to BTV infection, however, clinical outcome may vary remarkably. Indigenous African breeds of sheep are resistant and exhibit mild febrile reaction whereas the Merino and European mutton breeds are highly susceptible and frequently die. A marked variation in susceptibility is also encountered between different individuals of the same breed and even the same flock. The individual variation appears to be unrelated to previous exposure to homologous or related viruses and not clearly known at present. Luedke *et al.* (1964) observed that 5-year-old sheep were more susceptible than yearling lambs, as demonstrated by earlier onset of mouth lesions and longer clinical course. Severity of infection is also dependent on exposure to light. It is almost impossible to reproduce the severe disease by artificial inoculation in sheep kept in stables and not exposed to sunlight or UV light. In fact, it appears as if sheep infected with BTV develop some form of photosensitivity, which accounts for some of the severe signs seen under field conditions (Erasmus 1975).

The average incubation period following artificial inoculation of sheep

is 4-6 days, however, it may be as short as two days and occasionally as long as 15 days. The first sign of infection is almost invariably elevated body temperature, which may reach 107° F to 108° F. Luedke (1969a) reported that the mean peak viraemia in sheep was reached 6 or 7 days after infection. Peak viraemia of BT corresponds closely with peak temperature and leukopenia. The elevated temperature, which usually lasts 2 to 4 days, precedes marked depression and some times inappetance. The average febrile reaction is generally 6 days, although it may vary from 2-11 days. A good correlation between febrile reaction and severity of the clinical signs has been observed. During the period of high fever buccal mucosa and skin of the face become hyperaemic followed by erosions on the lips, dental pad and tongue. Nose is frequently occluded by a grayish- brown scab composed of desquamated epithelium and inspissated serum. The nasal septum is congested and excoriations are present on the muzzle. Coronitis resulting in lameness is also observed in many affected animals. Swelling of face and haemorrhage in upper and lower lips, dental pad, soft and hard palate is quite common. Acute disease is typically characterised by catarrhal inflammation of mucous membranes (mouth, nose and digestive tract) and the coronary band. Pain is evidenced by smacking of the lips and frequent movement of the tongue, resulting in slight frothing and also immersion of the mouth and lips in drinking water for long periods. The affected sheep also resent handling and examination of their mouths and find it difficult to eat. The tongue is frequently involved and initially may show intense hyperaemia with petechial haemorrhages of the papillae near the tip. It may later become grossly swollen and oedematous and may even protrude from the mouth. Occasionally in very severe cases, it may become cyanotic, hence the name bluetongue.

A watery nasal discharge is frequently present which later becomes mucopurulent and eventually dries to form crusts, causing severe dyspnoea. The respiration rate may increase and panting is frequently observed. In peracute cases alveolar oedema develops which leads to marked dyspnea and terminally froth may exude from the nostrils. The hyperaemia of the skin of the lips may extend to the entire face, ears and rest of the body. It is usually most pronounced in the groin, axilla, perineum and the lower limbs. Following an acute infection in sheep, extensive dermatitis occurs which causes abnormal wool growth, which evidenced by about 3-6 weeks later by a break in the wool that can lead to casting of the entire fleece.

Foot lesions usually develop with the subsidence of fever but occasionally during the peak of the febrile response. Initially hyperaemia of the coronary band is observed followed by petechial haemorrhages appearing under the periol, which later become streaky in appearance. The hind feet are more frequently affected and lesions are more pronounce on the bulbs of the feet, particularly on lateral hooves. Affected sheep are reluctant to walk due to pain, and are mostly recumbent or stand with the arch back and may even attempt walk on their knees. Within 7-14 days the horny laminae may start to separate from the sensitive laminae and clear break in the hoof develops. The old hoof gradually grows out and is sloughed completely about

3-4 months after infection. In some BT affected sheep rapid and extreme emaciation and weakness may be observed. Although this state can be partially ascribed to fasting and dehydration, it results mostly from very severe muscle degeneration and necrosis. Some sheep may develop torticollis, which can have serious consequences as the animals find it difficult to maintain their balance. Infection of pregnant ewes with BTV may lead to placentitis and active infection of the foetus leading to hydranencephaly and other congenital deformities may occur. The severity of lesions depends on the gestational age of the foetus at infection. Foetuses seem to be the most susceptible during the period of active brain development.

Recovery from the infection is dependent on severity of the condition. In mild cases recovery may be fast and uneventful. However, in severe cases death may take place in about 7-9 days. Mouth lesions normally heal quickly and extensive ulcerations may only leave a small scar after 7 days. Similarly, lesions on muzzle and nostrils disappear in about 5 days. Sheep with extensive muscle involvement may become weak and debilitated, and recovery could be protracted.

Clinical outbreaks of BT in sheep in India

A very severe outbreak of BT was recorded by the authors in Rambouillet sheep imported from Texas, USA in 1985, at the CSBF, Hisar. In the initial stages, the affected animals showed high-rise of the temperature ranging from 105°-108°F. Severely affected animals showed arched back (Fig. 16) and were disinclined to move. In acute cases, dramatic increase in respiration rate was recorded.

A nasal discharge at first watery and later mucopurulent and in some cases blood stained, appeared and eventually dried out to form crusts (Fig. 17).

Nose was often occluded by a grayish-

Fig. 16. Clinically bluetongue affected sheep showing classical arch back.

Fig. 17. BT affected sheep exhibiting blood stained and dried nasal discharge in the form of crusts.

Fig. 18. BT affected sheep exhibiting oedema of face.

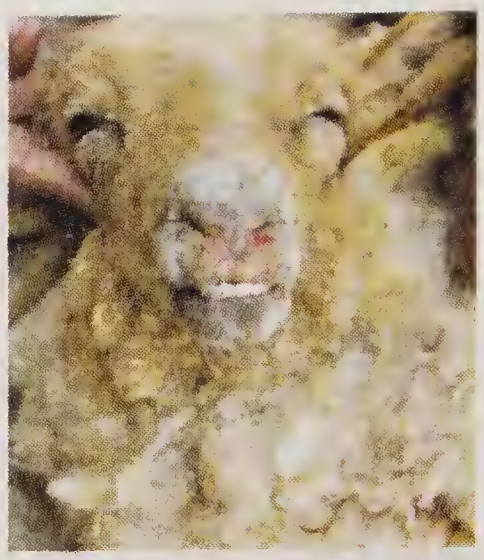

Fig. 19. BT affected sheep showing congested nasal septum and excoriations in muzzle

Fig. 20. BT affected sheep showing lesion on the tip of protruded tongue

brown scab composed of desquamated epithelium and inspissated serum along with excoriation of lower lip. Swelling of face, muzzle, nostrils and upper and lower lips (Fig. 18). Nasal septum was congested and excoriations on muzzle were also present (Fig. 19). In some cases tongue protruded out of mouth (Fig. 20).

Fig 21. BT affected sheep showing inflammation and reddening of coronary bands.

Excoriation of the epithelium of gums, the inner side of the lips and cheeks, the bars of the hard palate and the dental pad occurred. The careful examination of feet of the affected sheep revealed inflammation and reddening of coronary band (Fig. 22) and was observed more frequently in

Fig. 22. BT affected sheep showing cracking of skin.

hind feet. Due to inflammation of coronary band, the affected sheep showed lameness. There was no co-relation between oral and feet lesions. BT affected sheep some times also shows cracking of skin. It appears leathery in texture (Fig. 22)

Blood collected from BT affected Rambouillet sheep showing high rise of temperature (107° F to 108° F) and other typical BT clinical signs when inoculated intradermally and subcutaneously in crossbred sheep (50% Corriedale, 25% Merino and 25% Nali) produced mild BT evidenced by hyperemia of upper and lower lips (Fig. 23), pyrexia and isolation of BTV from blood of the experimentally inoculated sheep (Jain et al. 1986, Mehrotra and Shukla 1990).

Subsequently an outbreak of BT was reported from the Central Sheep and Wool Research Institute, Avikanagar, Rajasthan, in crossbred sheep. During this outbreak several hundred animals were affected. A second outbreak of BT was recorded in indigenous sheep in the unorganised sheep flocks in Churu district of Rajasthan. Young and the adult sheep were affected in this flock. Typical BT lesions were observed in the lambs between 1-2 months of age (Fig. 24).

The third outbreak of BT was reported from Government Sheep Breeding Farm Chittaurgarh, Rajasthan. In this outbreak also, the severity of the lesions was more in the exotic Rambouillet sheep as compared to indigenous stock.

Clinical BT outbreaks in exotic and indigenous breeds of sheep have also been reported from Gujarat, Maharashtra, Andhra Pradesh, Karnataka and Tamil Nadu. Mehrotra *et al.* (1991) reported a widespread outbreak of BT in Tamil Nadu during November and mid of December 1989. The disease was spread in two districts where almost 5,000 animals were exposed to

Fig. 23. Lesions in the upper lips of crossbred sheep experimentally infected with bluetongue virus.

Fig. 24. Lamb affected with BT showing mouth lesions.

disease. The clinical symptoms reported were rise in body temperature, depression, anorexia, oedema of face, conjunctivitis and nasal discharge. Oral mucus membranes had haemorrhagic oedema and few ulcers at dental pad. Owners because of poor carcass quality disposed off majority of affected animal. Cyanosis of tongue and facial oedema were pronounced in some animals.

Clinical BT in goats

Although the susceptibility of goats to BT was first described by Spreull (1905), surprisingly few reports are available on this animal. The degree of susceptibility of goats is quite variable; however, it is less susceptible than sheep. During the extensive outbreak of BT in Israel, Saanen breed of goats showed symptoms suggestive of BT (Komarov and Goldsmit 1951). Sapre (1964) reported outbreak of BT in which goats were also affected in Maharashtra, India. The symptoms reported were typical to BT and some of the infected goats died. Since the initial report of BT outbreak in goats in 1964, no other confirmed clinical BT case has been reported from India,

though presence of BTV antibodies in sera of apparently normal goats have been reported from several states of India (Prasad *et al.* 1992).

Luedke and Anakwenze (1972) infected Saanen breed of goats intradermally and subcutaneously, and reported that all the goats became infected as evidenced by development of leukopenia, isolation of the virus and presence of precipitating antibodies after 3 weeks of experimental infection. Thus, the goats are susceptible to BTV infection but the clinical disease rarely occurs. The goats might be playing an important role in epizootiology of BT in sheep by maintaining the virus along with other ruminant species.

Clinical BT in cattle and buffaloes

Infection of cattle with BTV was recognised as early as 1905 but until the description of BT in cattle by Bekker *et al.* (1934), BT was thought to be the frank disease of sheep only. Subsequently several workers have described clinical BT in cattle (Osburn *et al.* 1983). In the areas where BT is enzootic most infections are not clinically detectable. However, seroconversion and viraemias are main parameters to detect BTV infection in cattle.

The acute BT in cattle has been described to be characterised by initial pyrexia and stiffness of gait, followed by hyperaemia and subsequent ulceration of the nasal and oral cavities, which is accompanied by profuse salivation, crusting and sloughing of the muzzle, dermal oedema, vesicles and necrosis of the skin, severe coronitis and eventual alopecia. Mortality is usually low but convalescence may be prolonged, which may reduce milk production. A chronic ill-thrift syndrome associated with persistent BTV infection has been described. Bulls may also show temporary infertility due to initial infection, however, they recover subsequently. Studies on pathogenesis of BTV in cattle have been difficult because BT cannot be reproduced experimentally. The lesions seen in cattle indicated that the virus has predilection for the skin and mucosa of nasal cavity and upper gastrointestinal tract. In some natural outbreaks of BT older cattle are more commonly affected than young ones. Metcalf hypothesised that BT is a hypersensitivity disease, and the disproportionate incidence of older cattle was reflection of their increased likely hood of having experienced multiple exposures to BTV. Experimental evidence suggests that once the sensitised cattle are exposed to live BTV of the same serotype, the cattle develop clinical BT. The clinical signs appear to be the result of an IgE-mediated immune response characterised by weeping, ulcerative dermatitis, vesicular and ulcerative lesions of the oral cavity, coronet, and stiffness resulting in lameness (Osburn *et al.* 1983).

Bluetongue virus infection has been found associated with reproductive losses in cattle. The losses have been mainly attributed to abortions, foetal malformations and stillbirths. The foetal deformity due to BTV infection includes hydronencephaly and arthrogryposis, agnathia, prognathia and gingival hyperplasia (Osburn *et al.* 1983).

In India there are a variety of indigenous, crossbred and exotic breeds

of cattle which have been serologically found positive for presence of BTV infection from several states, however, no clinical BT has been reported so far from this country. Similarly several breeds of buffalo are reared in India for milk and draught purposes, which have been found positive for BTV specific antibodies in all the seasons (Jain *et al.* 1992, Mehrotra and Shukla 1990). Nevertheless, no clinical BT has been observed in buffaloes any where in the world. Probably the signs and symptoms of clinical BT are similar to other diseases like FMD, which is quite common in India. Hence, veterinarians might have failed to ascribe these signs to BT.

Even if clinical BT is not frequently encountered in cattle and buffalo, BTV infection in these species is important due to (i) outbreaks of BT (a) infection, (b) clinical disease in infected cattle; (ii) infected cattle and buffalo may be reservoirs of BTV; (iii) reproductive losses in pregnant cattle; and (iv) restrictions placed on importation and exportation of ruminants.

Clinical profile of the disease has been described to be variable in different breeds and species of domestic and wild ruminants. Besides ruminants, non-ruminant species such as canines and felines has also been reported to support the virus replication and cause clinical disease in certain situations. However, existence of clinical disease in a variety of wild ruminants still remains speculative. The clinical outcome of the BTV infection in different species of livestock has been changing like some other diseases. Therefore, the disease should not be confirmed only on the basis of clinical profile of the suspected animals but must be subjected to laboratory diagnosis.

Different serotypes of BTV have been found to cause variable clinical disease in the same species and breed of the animal. Such variations have also been noticed within the serotypes. Several factors are likely to be responsible for variable expression of clinical disease. Some breeds of sheep have been found highly susceptible and exhibit frank clinical disease. However, clinical disease is rarely observed in cattle and buffalo. In BT endemic regions, clinical cases of BTV are not frequently observed. The information is also lacking in the clinical disease in wildlife. Clinical disease management in small ruminants has to be developed. Treatment of BT affected animals should also be developed in the form of prescription. The clinical outbreaks of the disease need to be systematically studied and all the clinical signs and symptoms need to be minutely studied.

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8

Pathogenesis and Pathology

The ability of BTV strains/serotype to cause disease among susceptible domestic and wild ruminants depends on the pathogencity of the particular viral isolate, susceptibility of the host, vector species and a combination of environmental factors. The pathogenecity of the virus may have a broad spectrum. Theiler (1908) isolated BTV which had very mild pathogenecity. This isolate was further attenuated by serial passaging in sheep and subsequently used as vaccine in South Africa. Contrary to this isolate, Gambles (1949) got an isolate, which caused 60-70% mortality in sheep during severe epizootic in Cyprus in 1943. Neitz and Reimerschmid (1944) suggested that solar irradiation during the incubation and reactive period exaggerated clinical manifestations of the disease. Later it was observed that susceptible sheep inoculated with different isolates of BTV do not induce uniform clinical manifestations. This raises questions such as what intrinsic or extrinsic factors are responsible for type and severity of the clinical expression of the disease. The porphyrinemia is directly associated with hepatic insufficiency, hence, it may be possible to correlate the severity of the clinical response of BT infection with its proclivity to impair liver function under certain conditions. The possibility of heterologous antibodies in animals either to BT antigen or to closely related antigens, must be taken into consideration in determining the degree of virulence or avirulence.

Neitz (1948) reported that considerable variations in pathogenecity among several isolates of BTV were encountered when sheep were inoculated. Theiler and Vegilia isolates were of low pathogenecity, the Camp and Mimosa Park strains were more pathogenic, and the Bakker isolate was the most pathogenic. Theiler and Bekker's isolates were immunologically identical, but had wide latitude of pathogencity. Thus the question of what is responsible for the pathogenecity of BTV still remains unresolved.

Pathogenesis in sheep

Pathogenesis of BT is quite complex and depends on several factors including species and breed of animal, serotype/strain of the virus and species of the vector. Environmental factors may also affect the pathogenesis of BT. The studies conducted in the past over 20 years have made some progress in understanding the cellular and molecular mechanisms of transport of BTV from the site of the vector bite or experimental inoculation to the susceptible cells/tissues of the host. Following natural infection by the vector bite or

experimental intra-dermal inoculation, first site of the virus multiplication has been suggested to be regional lymphnodes and from the regional lymphnodes the virus spreads to other lymphnodes. The virus has very strong affinity to endothelium, periendothelial cells and pericytes of capillaries, precapillary arteriols and venules. The virus has also been observed in reticulo-endothelial cells of lymphnodes. On the basis of available data, it has been postulated that the virus first infects endothelial cells leading to cytoplasmic vesiculation, nuclear and cytoplasmic enlargement, pyknosis, karyorrhexis, swelling and necrosis as well as subsequent regenerative hyperplasia and hypertrophy of endothelium resulting in vascular occlusion, stasis and exudation (Stair 1968, Pini 1976, Lawman 1979).

Bekker *et al.* (1934) observed the development of lesions in tissues where mechanical stress is common in sheep naturally infected with BTV. The lesions were noticed in the oral cavity in proximity of teeth, muscular pillars and oesophageal groove of rumen. Thomas and Neitz (1947) confirmed these observations. The most severe lesions are invariably noticed in tissues (oral mucosa, dermis, hoof etc.) which have temperatures lower than internal tissues. This in turn results in hypoxia of over lying tissues with secondary lesions, particularly in stratified squamous epithelium. The severity of secondary lesions is greatly influenced by the site of the insect bite. Contrary to the belief that the virus first infects endothelial cells, lymphoid tissues may be the primary site of the virus replication. It has been suggested that entry of the virus may be an important determinant in primary distribution of BTV. If the virus enters the host by intravenous route, the primary site of the virus replication may be vascular endothelium while in intradermal entry of the virus lymphoid tissue may support the primary replication of the virus. Considering the fact that natural transmission of BTV occurs through intradermal route by *Culicoides* bite, the viral replication occurs in lymphoid tissues before general release in the blood stream. It has been demonstrated that the site of primary replication of the virus is lympho-reticular tissue. After viraemia, the virus could be detected in several tissues and organs of the infected host. The investigations have revealed that monocyte lineage cells are primary site of the viral replication, though the replication also occurs in endothelial cells and neutrophils. However, involvement of endothelial cells is highly selective. This suggests that there may not be generalized involvement of endothelian cells throughout the body.

Sheep naturally infected with BTV exhibit marked decline in erythrocyte counts and corresponding decrease in PCV and Hb values. In an experimental study, marked leukopenia was observed in the crossbred sheep inoculated with BTV serotype 1 isolated from Haryana (Chander *et al.* 1990). The depletion of leukocytes appears to be responsible for transient immunosuppression reported in BTV infected sheep (Ghalib *et al.* 1985). Haribabu (1985) studied the natural cases of BT in India and reported that there was marked decline in erythrocyte counts and corresponding decrease in PCV and Hb values suggesting the association of the virus with

erythrocytes resulting in lysis of erythrocytes. The author also reported that there was marked lymphocytopenia in BT affected sheep. The depletion of leukocytes may be responsible for immunosuppression reported in BTV infected sheep. Our recent *in vitro* studies on susceptibility of lymphocytes and monocytes to BTV 1 infection have suggested that the replication of the virus occurs in these cells (Garg and Prasad 1994, Garg and Prasad 1995)(Fig. 25). Ravindran (2003) investigated pathogenesis of BTV 18 in an experimentally inoculated sheep. Microscopically interstitial pneumonia was observed in all the infected sheep. The study also indicated mild leucopenia and decline in PCV values. It was concluded that apoptosis is not primary mechanism of BTV pathogenesis.

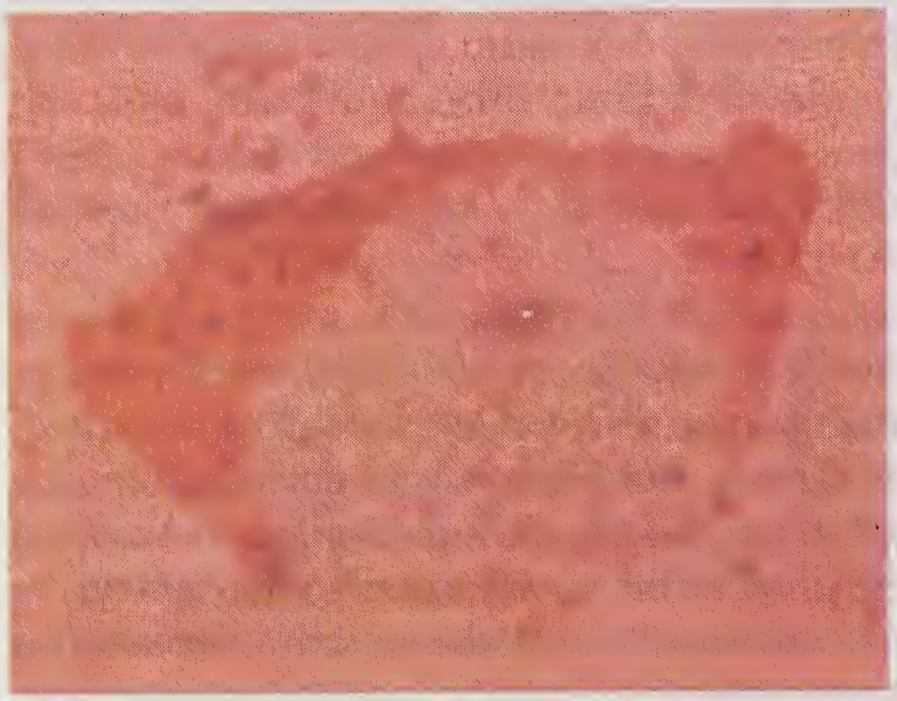

Fig. 25. BTV infected cattle lymphocyte stained by immunoperoxidase procedure.

Mehrotra *et al.* (1995) reported a concurrent outbreak of BT and morbillivirus (PPRV) in sheep. Five outbreaks of BT were reported from Madhya Pradesh, Maharashtra, Tamil Nadu, Jammu & Kashmir and Uttar Pradesh. The morbidity and mortality was reported among indigenous and exotic breeds of sheep. In all outbreaks distinct clinical disease was reported (Mehrotra *et al.* 1996).

Infection of pregnant ewes with BTV may lead to placentitis and active infection of the fetus. Depending upon the stage of gestation, the outcome of the BTV infection may be death of the fetus or development of fetal abnormalities such as encephalopathy (dummy lamb) of skeletal system (Griner *et al.* 1964). Srivastava *et al.* (1989) conducted an extensive retrospective study in India on the teratogenic effect of BTV infection in pregnant sheep in Central Sheep and Wool Research Institute, Avikanagar, Rajasthan, where BTV is endemic since 1980. They reported a wide variety of congenital defects including suicephally bifid tongue, lateral oral openings, absence of pelvic girdle, rectum, sex organs, hind legs and abdominal muscles. Sharma *et al.* (1985), further opined that frank clinical cases of BT occur in this farm during August-October each year, a major breeding season of the flock. Thus they established the relationship between the occurrence of congenital defects due to BTV and the period of pregnancy.

Pathogenesis of BTV infection in cattle

The frequency of infection of cattle with BTV is generally higher than in sheep, but frank disease in cattle is rare. Similarly, experimental

production of disease with virulent virus rarely succeeds. Pathogenesis of BT is poorly defined in cattle. However, it has been suggested that the disease expression is associated with an IgE-mediated hypersensitivity reaction induced by previous exposure to BTV or related orbiviruses. The appearance of symptoms and lesions is paralled by rapid elevation of serum IgE-specific BTV antibodies, which in turn is associated with release of endogenous mediators histamine, prostaglandins and thromboxane A_2 (Emau *et al.* 1984, Anderson *et al.* 1985).

Bluetongue virus infection of cattle is common in endemic regions and usually inapparent. The infection is generally characterised by prolonged viraemia, which is of major importance, as it increases the likelihood of infection of insect vector after feeding on infected cattle. Cattle have been postulated as important player in the over-wintering mechanism of the virus. Several possible reasons for prolonged viraemia have been investigated. Lack of an immunologic response to the virus is not responsible; becauses BTV elicits a prompt and sustained humoral immune response in cattle. The neutralising antibodies may be detected in the serum as early as 14 days after inoculation of calves (MacLachlan *et al.* 1987, Barratt-Boyes and MacLachlan 1994).

BTV has been found highly cell associated during viraemia and there is strong evidence to suggest that the cellular association protects circulating virus from elimination by neutralising antibodies during prolonged infection. During later stages of viraemia in calves, BTV is exclusively associated with RBC fraction of blood. The virus can be consistently isolated from these cells up to and even beyond 40 days after inoculation, despite high titres of neutralising antibodies in serum. The electron microscopic investigations have indicated that BTV is present in the invaginating RBC membrane. Red blood cells are not productively infected due to lack of necessary cellular machinery. Immunogold labeling studies have suggested that virions present in the invagination of RBC membranes are not accessible to antibodies (Brewer and MacLachlan 1992). Natural history of experimentally induced BTV infection of cattle has been described by several workers (MacLachlan *et al.* 1987, Barratt-Boyes and MacLachlan 1994, MacLachlan *et al.* 1990) and depicted in the Fig. 26 (Barratt-Boyes and MacLachlan 1995). These

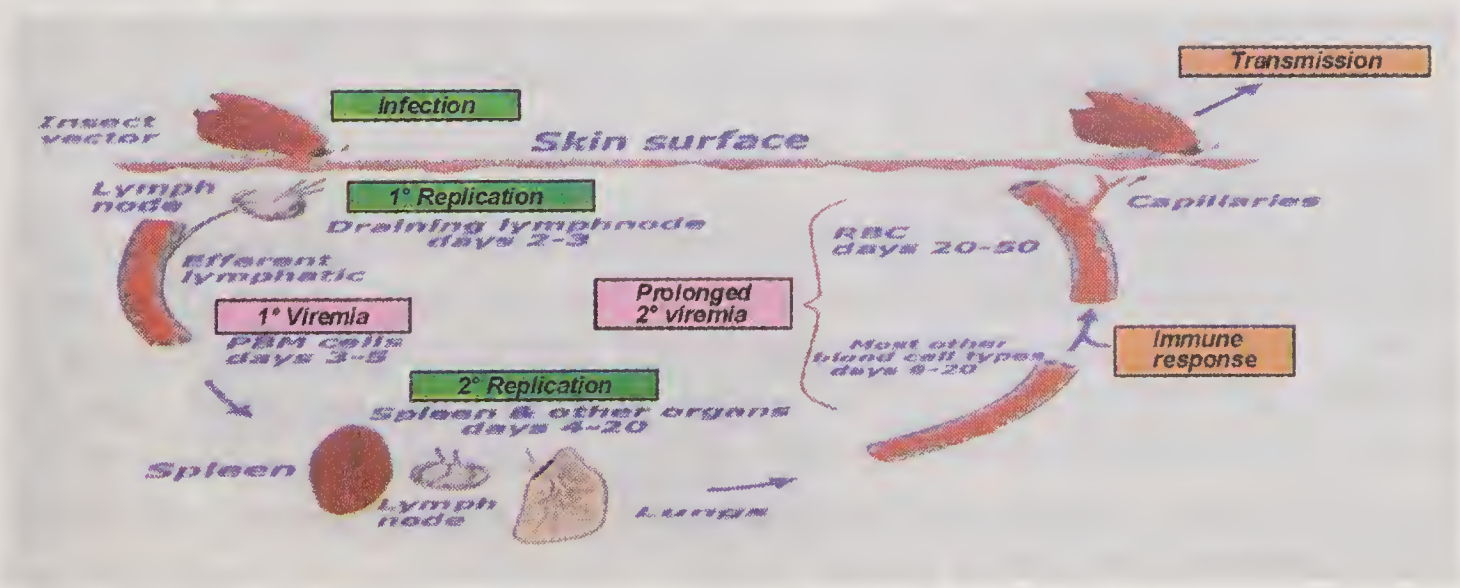

Fig 26. Diagramatic representation of natual cycle of bluetongue virus in cattle.

workers used lymphatic canulation to study the pathogenesis of BTV in cattle and suggested that primary viral replication occurs in regional lymphnodes draining inoculated skin. Then the virus enters the circulation via infected cells in the efferent lymphatic vessels. Peripheral blood mononuclear cells, the first blood cells from which the virus can be isolated, carry virus to secondary sites of replication. *In vitro* studies have suggested that monocytes may be the principal cells affected in the blood. The virus can be isolated from most tissues during infection, but secondary replication takes place primarily in spleen and lymphnodes as determined by virus isolation studies. Virus release into the circulation results in secondary viraemia when virus can be isolated from most of blood cell fractions. Bluetongue virus invasion of blood cells during secondary viraemia is probably non-selective, because cells with highest concentration in blood (RBC and platelets) consistently yield the highest titers of virus. The RBCs and platelets are likely to be invaded by the virus when they are mature cells in the circulation and not when they are precursor cells in the bone marrow, because BTV is first isolated from bone marrow several days after the onset of viraemia and only a low titre is measured (Barratt-Boyes and MacLachlan 1994, MacLachlan *et al*. 1990). The appearance of neutralising antibodies in the circulation coincides with loss of the virus from most blood cell fractions except RBC. Red blood cells containing the viruses are likely to circulate for their normal life-span, because viral RNA can be detected in blood cells by PCR amplification up to 140 days post experimental inoculation of cattle (MacLachlan *et al*. 1994). Similarly, BTV RNA was detected in blood of naturally infected cattle for 160 days by PCR amplification whereas virus was isolated for considerably shorter period (Katz *et al*. 1994). After removal of all blood cells containing virus (probably through normal interaction of senescent RBC with the mononuclear phagocytic system) infection is terminated. There is no evidence for the long-term persistence of BTV, after natural or experimentally induced BTV infection (Barratt-Boyes and MacLachlan 1994, Katz *et al*. 1994, MacLachlan *et al*. 1990, MacLachlan *et al*. 1994). Furthermore, the viral nucleic acid, which has been detected in blood cells but not the virus, has been non-infectious to both sheep and insect vector. This indicates that cattle that contain the viral nucleic acid but not the infectious virus are unimportant to the natural cycle of BTV infection.

Postmortem observations

The lesions in sheep died of BT are often dependent on the severity of the disease, and the stage of the disease when the death occurs. The most prominent lesions are usually located in and around mouth. The mucosa is oedematous, hyperemic and sometimes cyanotic and petechiae may be present. Excoriations are usually seen on lips, dental pad (Fig. 27), the sides, tip and anterior dorsum of the tongue and also on interior surface of the cheeks opposite the molar teeth.

The excoriations are covered with grey necrotic tissues. Trachea of

BT affected sheep may show extensive haemorrhages (Fig 28.). Mehrotra *et al.* (1991) reported a widespread outbreak of BT in Tamil Nadu. Oral mucus membrane had haemorrhagic oedema and few ulcers at the dental pad. In two cases cyanosis of the tongue was also seen. Recovered sheep manifested debility, loss of appetite, falling of wool/hair and torticollis.

Fig. 27. Dental pad showing excoriations after infection of BTV

Hyperemia of ruminal papillae, ruminal pillars and reticular fold is commonly observed (Fig. 29). Petechial haemorrhages are frequently present in mucosa of the abomasums and in the gall bladder (Erasmus 1975).

Nose is frequently occluded by a greyish- brown scab composed of desquamated epithelium and inspissated serum. The nasal septum is congested and excoriations are present on the muzzle. The lungs may be edematous and pneumonia is commonly observed due to inhalation of ingesta. Changes in the cardio-vascular system are characterised by widespread hyperemia, oedema and haemorrhages. Distinct hemorrhages are consistently found in tunica media at the base of pulmonary artery and are generally considered as pathognomonic of BT. Petechiae and ecchymotic haemorrhages may be found in the myocardium, epicardium and endocardium. Haemorrhages are often observed particularly near apex of the heart (Erasmus 1975). The lymphnodes, particularly those draining tissues of the head are commonly enlarged. The spleen may be slightly enlarged with sub-capsular petechial haemorrhages. Kidneys are generally congested and petechial hemorrhages (Fig. 30) may be present in the mucosa of urinary bladder, urethra, vulva or penis sheath (Erasmus 1975).

Fig. 28. Trachea showing extensive haemorrhages

Fig. 29. Extensive petechial haemorrhages in rumen of the affected sheep

Overspread haemorrhages are consistenly noticed in peripyloric musculature at the junction of the duodenum and abomasum. Congestion and haemorrhages are also observed around thyroid glands. The lymphnodes of head, especially the retropharyngeal and submandibular are often markedly enlarged, oedematus and haemorrhagic. Other lymphnodes of head, neck and thorax are less severely involved. Tissues of retropharyngeal and paranasal sinuses are often congested.

The skin often shows intense hyperemia, particularly in the areas not covered by wool. The vascular network on inside of the skin is generally markedly congested and even the smallest blood vessels become very conspicuous. The animals that die later than 14 days, often show degeneration and necrosis of skeletal musculature, resulting in sever wasting of carcass. Individual muscle fibres or entire muscles lose their pigmentation and the inter-muscular fasciae are infiltrated with a clear fluid, resulting in gelatinous appearance. The cause of death in such animals is not clear but it could be due to general exhaustion. Histological examination does not reveal any pathognomonic changes and generally confirms the gross pathological findings. Stair (1968) gave a detailed description. The most frequently observed lesions BTV infected sheep are located in digestive system. The mouth lesions are characterized by excoriation of

Fig. 30. Kidney showing haemorrhages.

the lips, tongue, dental pad, buccal mucosa and gums. The oesophagous is congested, ulcerated and petechiated and there are ecchymotic haemorrhages on the pillars of the rumen. The remainder of the digestive tract may exhibit vascular congestion and haemorrhages and, in more severe cases an extensive catarrhal process may extend into the large intestine. Microscopic examination of mucosal lesions shows mononuclear cell infiltration and necrosis of epithelial cells in which large acidophilic intracytoplasmic masses accumulate. Infiltration by neutrophils, macrophages and lymphocytes is present in acute cases (Verwoerd and Erasmus 1994).

Course of disease

Several factors including virulence of the serotype involved, species/breed of susceptible host and the management of the affected animal determine the duration of the disease. The variations observed in viraemia in experimentally inoculated sheep, goat and cattle are presented in Table 22. The affected animals showing mild signs quite often recover within a week. However, acutely affected cases take longer time to recover and many of them die due to pneumonia and secondary infections. The recovered animals become very debilitated and weak. The mortality rate in BTV is highly variable and depends upon a wide variety of factors. It has been observed that death usually occurs within 1-10 days after appearance of the visible signs.

Most of the information regarding pathogenesis and gross pathological lesions has been derived from the studies conducted on field out breaks of the disease. The efforts to produce frank clinical disease in experimentally infected sheep, which is the most susceptible animal host for BTV, have been largely unsuccessful. The studies have suggested that BTV particles are

Table 22. Different duration of viraemia observed in experimentally inoculated sheep and goats

Animal species/breed	BTV serotype	Duration of viraemia (days, PI)	Reference
Sheep			
Warhill	Wyoming isolate	1st-31st	Luedke (1969)
Merino	4, 10, 16	2nd-26th	Goldsmit *et al.* (1975)
Corriedale	10, 11, 13, 17	3rd-1st	Ghalib *et al.* (1984-85)
Lesboss	4	3rd-54th	Koumbati *et al.* (1999)
Friesian	4	3rd-31st	Koumbati *et al.* (1999)
Goats			
Indigenous Israel goats	4	2nd-19th	Barzili *et al.* (1971)
American strains of Saanen	8	1st-21st	Luedke and Anakwenze (1972)
Saanen	4	3rd-34th	Koumbati *et al.* (1999)
Lesboss	4	3rd-54th	Koumbati *et al.* (1999)

enzymatically modified in the insect vectors and this change is critical for inducing frank clinical disease in the susceptible host. The ambiguities still persist in the literature regarding the longevity of the persistence of the virus in the infected animals. Past studies have indicated that there are different types of target cells in mammalian hosts. Molecular pathological studies are required to understanding mechanism of pathogenesis of BTV. Molecular studies would also help in understanding variation between and within serotypes vis-á-vis different breeds of sheep. The lack of good animal model system has been major impediment in understanding the molecular basis of pathogenesis.

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9

Impact of Bluetongue on Animal Reproduction

Several infectious diseases of livestock have been shown to cause great reproductive losses. Some of the infectious agents may be transmitted from mother to foetus *in utero* while others through contaminated semen and embryo transfer. Artificial insemination was introduced more than 50 years ago to distribute useful genes and eliminate chances of transmission of venereal diseases. However, precautions must be taken to ensure that artificial insemination does not spread pathogenic agents. It has been recorded that a single bull may yield up to 1,000 doses of semen for artificial insemination from one ejaculate that are widely distributed nationally and internationally. Therefore, potential for spread of infectious diseases through semen is significant. The freezing conditions used for preservation of semen enables several infectious agents to survive. Hence, the infectious agents may be easily transmitted through artificial insemination. Considering the risk of transmission of diseases through semen, strict health monitoring and surveillance of bulls and rams is required to produce pathogen free semen. The OIE (1986) has recommended certain guide lines to be followed internationally in this regard. The clear understanding of natural history of a disease will determine the timing of the test in relation to semen production. The classical serological diagnostic tests have limitations such as lack of sensitivity and specificity. Therefore, more sensitive and specific newer diagnostic procedures such as PCR and DNA probes need to be utilised for screening of the animals as well as their germplasm. The past experience has clearly demonstrated potential risk associated with animals and their germplasm tested by conventional serology. However, the movement of embryos and subsequent transfer to the recipients is relatively less hazardous as compared to semen.

There are quite a few facets of BT which are still not clearly understood. Transmission of BTV is undoubtedly through *Culicoides* midges, but there are many aspects of mechanism of transmission that remain to be understood. For example, it is not known whether transmission of BTV occurs in the absence of *Culicoides* vector or not. The presence of BTV in secretions and excretions including semen has been demonstrated under certain circumstances but the underlying factors remain enigmatic. Though the virus passes through the placenta of sheep, it may or may not do so in

cattle. There is lack of information on the nature and extent of serological response to infection, which may be absent or undetectable in some animals of the population. Therefore, cost-benefit analysis of importing animals or their semen or ova or embryo becomes very difficult. This chapter describes current understanding of transplacental transmission of BTV to foetus and transmission through semen and embryos.

Transplacental transmission

Transplacental transmission of BTV has been widely studied during the past more than 30 years resulting in accumulation of large scientific data but the understanding on the transplacental transmission of BTV in different species of ruminants is still not clear.

A consequence of BTV infection in pregnant sheep and cattle may be the transplacental transmission of the virus to the conceptus. The subsequent outcome may depend upon the virulence of the infecting virus, source and concentration of the inoculum and age of the embryo when the infection occurs in the pregnant animal. A developing embryo (13-45 days of sheep) or foetus of cattle (46 days to term) may become infected through the placenta which may not act as a barrier to BTV. Instead, the placenta may serve as susceptible biological tissue junction that may support BTV replication. In some circumstances, BTV crosses this junction and the foetus is infected and subsequently, the foetus dies and expelled from the uterus. In another situation, the embryo or foetus may recover and be deformed, or the foetus may survive and carried until it is born. In the latter situation, some offspring may be immunologically unresponsive to BTV and harbour the virus throughout life. Non-lethal congenital infections are more troublesome and are of major threat to livestock industry because of their potential for life long persistence of the virus in such animals.

The outcome of *in utero* infection of BTV depends upon the gestational age. Infection in the first trimester may result in foetal death. As gestation age increases, infection may result in abortion, stillbirths, birth of dummy or deformed animals. The other congenital defects including suicephally, bifid tongue, lateral oral openings, and absence of pelvic girdle, rectum, sex organs, hind legs and abdominal muscles have also been observed due to transplacental transmission of BTV. It has been observed that laboratory attenuated BTV strains/serotypes used for vaccine production cross placenta when pregnant ewes are vaccinated with such viruses.

The data accumulated so far on the different aspects of BTV infection in relation to reproduction is obviously confusing and contradictory in both sheep and cattle. Many factors including (i) the source and serotype of the virus, (ii) the age of the foetus, (iii) breed and species of the animal, (iv) the environment, and (v) interaction with other agents may influence the consequences of infection, particularly with respect to vertical transmission. The variations observed in the transplacental transmission of BTV in cattle and sheep is possibly due the above factors. Though there are conflicting reports, the experts agree that there is no sound scientific evidence to

suggest that natural BTV (field strains which have not been attenuated in the laboratory) crosses the placenta to cause foetal infection and production of persistently infected, seronegative adult animals (OIE 1992). The observations for and against transplacental transmission of BTV in sheep and cattle are presented.

Sheep: The evidence for transplacental transmission of BTV in sheep is more convincing than that in cattle. Schultz and DaLay (1955) described losses in new-born lambs associated with use of live BTV vaccine in pregnant ewes. Retrospective examination of the data indicated that the incidence was highest in lambs of ewes vaccinated between 35 and 42 days of gestation. Hydranencephaly was seen in the more severely affected lambs. Subsequently, other researchers showed that attenuated BTV vaccines caused the cerebral malformations. However, the lesions and the type of anomaly depended on the age of the foetus at the time of vaccination of the pregnant ewes (Young and Cordy 1964, Osburn *et al.* 1971a, b). Anderson and Jensen (1969) in an experimental study showed that transplacental transmission of BTV occurred in 30% of ewes inoculated at either fifth or sixth week of gestation. In another study, sheep were infected with BTV between 60 and 90 days of gestation and found transplacental infection of the foetus was common (Gibbs *et al.* 1979). The virus was detected in the blood of 80% of the new-born lambs during the first week of life, even though colostrum has been supplied from the mother. In some lambs viraemia ceased before 28 days, however, in others the virus remained detectable after even 2 months. Nevertheless, contrary to the evidence for transplacental transmission of BTV, it was shown that 10 ewes inoculated with BTV serotypes 20, after 35-42 days of service, produced normal lambs (Flanagan *et al.* 1982). The researchers did not find any evidence of transplacental transmission of the virus. The findings of Gibbs *et al.* (1979) related to transplacental transmission of the virus could not be repeated (Richardson *et al.* 1985). It appears that there is no concrete scientific reproducible data to strongly support transplacental transmission of the virus in sheep.

Cattle: The information available on the possibility of transplacental transmission of BTV in cattle has been summarised in Table 23. Isolation of BTV from the spleen of aborted bovine foetus provided first evidence for the possibility of transplacental transmission of the virus (Bowne *et al.* 1968). Evidence for transplacental transmission of BTV was also provided by McKercher *et al.* (1970) where an association with congenital hydranencephaly was reported. Abortions and development associated abnormalities in calves have been reported in cattle herds endemically infected with BTV. Later, infection of cattle as foetuses with BTV was described. It was demonstrated that heifers infected during early gestation could transmit the virus transplacentally to the foetus with development of persistent infection (Luedke *et al.* 1977). Several reports have suggested that early foetal infection with BTV results in severe nervous system damage and do not lead to production of normal calves (Table 24). Liendo and Castro (1981) have also reported abortions and anomalies in cattle herds infected

Table 23. Evidence for transplacental transmission of bluetongue virus

Evidence	Reference
Isolation of BTV from spleen of an aborted foetus	Bowne *et al.* (1968)
Isolation of BTV from 6 months old bovine foetus	Luedke *et al.* (1970)
The inoculation of 5 months and 7.5 months old foetuses with BTV resulted in infection of the foetuses and induction of specific antibody response	Jochim *et al.* (1974)
Exposure of pregnant heifers at 60 and 120 days of gestation through bites of infected *Culicoides* midges resulted in abortion of two animals (one from each group). One pregnant animal produced stillborn calf and the other seven pregnant animals gave birth to calves that had various degrees of gross dysfunction. Four of the eight calves yielded virus in their blood at birth. Three of these calves were immunologically incompetent.	Luedke *et al.* (1977)
Heifers, served by a bull known to shed BTV in his semen, yielded calves with varying degree of congenital abnormalities and viraemia.	Luedke and Walton (1981)
Demonstration of secretion of BTV in semen of bull exhibiting persistent viraemia for 11 years	Luedke *et al.* (1983)
Described bull that sporadically shed BTV in semen for several years that remained serologically negative	Schultz *et al.* (1985)

Adopted from Roberts *et al.* (1993).

with BTV and isolation of the virus from blood and bone marrow of several neonates and foetuses.

However, there are a few contradictory reports which provide evidence against the transplacental transmission of BTV in cattle. MacLachlan *et al.* (1985) have pointed out that central nervous system (CNS) defects are not many in BTV endemic areas and virus does not cause significant reproductive losses. In an experimental study it was shown that inoculation of pregnant cows at different days of gestation did not result in infection of the foetus (Parsonson *et al.* 1987). The foetuses did not show any CNS pathology reported by some workers earlier. Inoculation of pregnant cows at either 40 or 60 days of gestation with BTV did not result in infection of the foetus and gave birth to normal calves without any evidence of BTV infection (Roeder *et al.* 1991, Thomas *et al.* 1986).

Shedding of bluetongue virus in semen

In an experimental study, BTV was isolated from 19 out of 181 ejaculates collected from 20 infected bulls during the period of viraemia (Bowen *et al.* 1985). However, the virus was never isolated from 13 of the 20 bulls. The study suggested that seminal shedding of the virus closely followed peak virus titre in the blood. The nine heifers inseminated with BTV positive semen, six became pregnant and three became viraemic and seroconverted while one heifer developed antibodies but did not become detectable viraemic. Later, Gard *et al.* (1989) exposed a small group of bulls

Table 24. Summary of results from various studies of the outcome of deliberate inoculation of BTV into bovine foetus

BTV serotype	Virus titre	Route of inoculation	Foetal age (days)	Foetal serological response	Foetal lesions	References
10	5×10^3 $TCID_{50}$	Foetus	126	N R	Cow aborted at 262 days	Barnard and Pienaar (1976)
					Hydranencephaly	
10			138		Hydranencephaly	
10	5×10^6 $TCID_{50}$	Foetus	125	+	Hydranencephaly	MacLachlan and Osburn (1983)
10	5×10^6 $TCID_{50}$	Foetus	125	+	Not reported	MacLachlan *et al.* (1984)
10	N R	Foetus	125	+	Hydranencephaly	MacLachlan *et al.* (1985)
10, 13, 17	N R	Foetus	85-90		Aborted	
		Foetus	85-90	+	Hydranencephaly	
11	10^3pfu	Foetus	106	-		Thomas *et al.* (1986)
			113	-	Central nervous	
			122	+	system damage	
11	N R	Foetus	120		Cerebral lesions	Waldvogel *et al.* (1989)
11	N R	Foetus	243	+	No lesions	
11 (two strains)	10^4 pfu	Foetus	120	+	Cerebral lesions	Waldvogel *et al.* (1992)

Adopted from Roberts *et al.* (1993); NR, Not reported.

to natural infection with arboviruses. The bulls were bled and ejaculated regularly and semen and blood were processed for the virus isolation. This study indicated that 29 exposed bulls had experienced 79 viraemic episodes over the period of five years. In no instance was there unequivocal evidence of BTV presence in semen. However, contrary to this observation, isolation of virus from the bull infected *in utero* has been described (Luedke *et al.* 1983, Luedke and Walton 1981). These workers described shedding of BTV in semen of the persistently infected bulls. Schultz *et al.* (1985) also reported sporadic isolation of BTV from semen of the bull which remained negative serologically. However, the work of Luedke and his associates has not been confirmed.

Transmission of BTV by embryo transfer

For an infectious disease to be transmitted via an embryo, the pathogen has to be transferred either in embryo, in or on the zona pellucida of the embryo, or in the fluids in which the embryo is transferred (International Embryo Transfer Society 1987). In recent years a number of studies have been carried out to determine the potential of early embryos to transmit BTV. Thomas *et al.* (1985) carried out three types of experiments in cattle:

i. Bovine embryos were exposed *in vitro* to different amounts of BTV (10^2-10^7 infectious units/ml), incubated for 1 or 24 hours, washed, further incubated for 24 or 48 hours and assayed. From 116 embryos, no BTV was recovered in this study.
ii. Embryos from BTV viraemic donors were transferred into uninfected recipients. None of the recipients seroconverted nor was antibody or virus detected in calves which resulted.
iii. Semen containing BTV was used to inseminate four super ovulated heifers, two of which subsequently developed viraemia. Embryos were recovered from donor cattle and transferred to 16 uninfected recipients, of which nine became pregnant. None of these seroconverted by the fourth month of pregnancy.

Embryos in the above experiments were washed 10-times and zona pellucida was intact. Subsequently, Acree *et al.* (1991) reported that there was no evidence of transmission of BTV when 231 embryos taken from acute and convalescent experimentally infected heifers were transferred to the susceptible recipients.

Based on the published reports, there is general consensus at this time that while infected semen can infect susceptible cows and heifers and produce viraemia, BTV is not present in embryos resulting from fertilization of ova with infected semen or from heifers viraemic at ovulation. Therefore, it appears that embryo transfer, if conducted correctly, in accordance with the procedure recommended by International Embryo Transfer Society (1987), substantially reduces the risk of BTV transmission when compared with movement of breeding livestock (Gibbs and Greiner 1988).

Protocol for certification of semen for export/import: The need for genetic improvement of livestock has led to international movement of germplasm.

However, semen is a potential mode of transmission of certain viruses. Therefore, extensive testing of semen for international exchange is mandatory. Bluetongue virus is prevalent in several regions of the world. Hence, semen must be tested and certified as free of BTV before approval for import/export.

Initially criterion for selection of donor bulls was based on the condition that they were BTV antibodies negative on complement fixation or agar gel immuno-diffusion (AGID) tests. This approach for selection of bulls suffers from a number of drawbacks. For example, this would exclude the valuable bulls which had history of BTV infection if they were no longer carriers of the virus, at the same time there is risk of accepting animals with an acute infection or latent carriers with no detectable antibodies (Ianconescu and Kaufman 1992). Therefore, new more comprehensive testing procedures have been recommended for certification. In 1983, a protocol for certification of semen for export was presented which was adopted by several countries with slight modifications (Osburn *et al.* 1983). According to this procedure even the antibody positive bulls could be certified as free of active BTV infection. Subsequently, Kimron Veterinary Institute (KVI) protocol (1986) and Animal Health Certificate (AHC) for importation of bovine semen of the Veterinary Services of Netherlands (1989) were developed. Swayer *et al.* (1992) presented a protocol modified from Osburn *et al.* (1983) protocol. According to this protocol, bull blood, not the semen, is optimal sample for virus amplification in sheep. BTV if present in semen would be present in blood at much higher titre than that in semen. Further more, BTV would be detected in blood much longer than that in semen. Therefore, it was suggested that weekly blood collection is sufficient as the viraemia in cattle may be of 8 weeks. Inoculation of blood directly into chicken embryo is redundant. Inoculation of bull blood directly into seronegative sheep is preferred for amplification. Seroconversion of sheep is more sensitive detection method (Sawyer *et al.* 1992). These workers recommended that group specific cELISA should replace AGID test since AGID is less sensitive than cELISA. Non-structural protein antibody test and serotype specific cELISA have been recommended in order to detect active infection.

Collection of samples from donor bulls

Blood, semen and serum samples should be collected from the donor bulls and processed as described below:

Blood samples: Heparinized blood samples should be collected from the bulls at weekly intervals beginning with the first day of semen collection and including on final blood sample at the conclusion of semen collection period. The AHC protocol requires collection of two blood samples per week during the entire semen collection period. The blood samples should be stored at – 40°C until the initiation of 28 days testing period.

Serum samples: Serum samples should be collected from the bulls on the same day as blood samples. At vector season additional samples should be collected at 2 weeks and 4 weeks after the semen collection.

Testing of samples

Testing of blood, semen and serum is done by serological and virological assays to detect virus and antibodies. The procedure for testing of each sample is described here.

Blood: Blood samples should be inoculated into sero-negative susceptible sheep and the inoculated sheep should be observed for development of clinical signs such as rise in body temperature for at least 4 weeks. The blood and serum samples should be collected from the inoculated sheep twice a week for 3 weeks and subsequently once a week for another four weeks. The blood samples from the inoculated sheep should be tested in embryonated chicken eggs for detection of virus (Ianconscu and Kaufman 1992).

Semen: Semen is not suitable for egg as well as cell culture inoculation owing to very high toxicity. Therefore, the semen samples can be directly inoculated in sheep. In this host semen may cause local or generalised allergic reactions even in absence of viral contamination. The modified protocols of Sawyer *et al.* (1992) eliminate the need for testing of semen due to the reasons described in this chapter.

Serum samples: The serum samples collected from donor bulls as well as experimentally inoculated sheep should be tested by group specific cELISA and evidence of active virus infection by serum neutralisation test (indicated by 4-fold rise in titre to any serotype of the virus).

When the experimentally inoculated sheep yields negative results serologically as well as virologically, the bull semen can be certified as free of BTV.

Regulatory issues

Protection of health status of national livestock herds and flocks and wildlife and safe guarding against introduction of infectious agents, which can cause serious economic loss to the livestock and food industries, is the prime responsibility of regulatory authorities of different countries. However, it is well known that improvement in the performance of indigenous stock can be enhanced by international exchange of superior ruminant genetic material. Conservation of certain rare wildlife species may also be affected by exchange of germplasm between the countries. It is believed that totally safe import policy is not possible. However, this is unrealistic, and, therefore, import policies of the nations must be based on risk assessment. The International Working Group on Regulatory Issues (OIE, International Working Group,Team Report 1992) has recommended that following points must be kept in mind by the germplasm importing countries:

i. The distribution, level and seasonality of incidence of the infectious agent in the exporting country.
ii. The presence of vectors or potential vectors in the importing country and the seasonality of their incidence.
iii. The presence of susceptible hosts in the importing country and their relationship to potential vectors.

Certain species of *Culicoides* have been identified as competent vectors of BTV in certain geographic regions of the world. However, there is lack of information about other species of *Culicoides* to act as potential vectors of BTV. The past studies have very strongly indicated that BTV can be shed in semen only during viraemic phase. Convincing scientific evidence is still not available to suggest that naturally occurring BTV ever crosses placenta and causes foetal infection and production of persistently infected seronegative adult animals.

Movement of live animals

Transport of live animals represents greater risk than movement of semen, embryos and other animal products. However, this issue must be addressed and the risk assessment should be made objectively based on the sound scientific data currently available. It is reasonably safe to move seronegative ruminant livestock from a seronegative population to a BTV negative recipient zone. However, seronegative animals from seropositive herds and flocks need to be checked by sensitive tests to ensure that they are not incubating the virus. The seropositive animals should be imported only in rare situation after ascertaining that the animals are not viraemic and are not carrying the virus.

Movement of semen

The International Working Team on Germplasm has recommended that the bulls used for artificial insemination (AI) should be from BTV-free areas and should be regularly tested serologically as well virologically. If the bulls are from BTV endemic areas, the testing should be done rigorously again after 35 days. If they are negative, their semen should be cleared for export after completion of the semen certification protocol described in this chapter.

Movement of embryos

The International Working Team on Germplasm agrees with conclusions of International Embryo Transfer Society (IETS) that the risk of BTV transmission through embryo transfer is negligible, provided that embryos are appropriately handled after collection (Rev. Sci. Tech. Off. Int. Epiz. 8 (2), 1989). Any manipulation of the embryos for sexing, splitting, and similar procedures should be carried out after these IETS-endorsed procedures. The embryo donor animals must be tested the same ways as bulls for semen export.

Movement of foetal bovine serum

Foetal bovine serum (FBS) is used extensively throughout the world as an important ingredient of media for cultivation of vertebrate as well as invertebrate cell cultures. Therefore, FBS has a substantial global commercial significance. Serious concern has been raised about its contamination with viruses and/or antibodies. Concern for the purity and quality of FBS becomes

more important when it is to be used for production of vaccines and other biological products through cell cultures. Bovine viral diarrhoea virus has been found in FBS. Bluetongue virus and other Orbiviruses may also have potential to contaminate batches of FBS. Currently WHO recommendations for quality control of FBS are followed (WHO Technical Report Series 673). This requires testing of batches of FBS in bovine cell cultures with subpassaging, observing CPE and then carrying out specific tests for certain viruses. It would be quite possible to eliminate BTV contaminants by physiochemical treatment such as irradiation or by gentle acidification of FBS and then readjustment to normal pH. However, these treatments have been found to affect nutritional value of FBS adversely. Therefore, direct tests should be made on FBS using modern and sensitive tests available for detection of BTV.

Meat, wool, hides and other products

There is no evidence to support the imposition of any BTV testing requirements or restrictions on the movement of meat, wool, hides and other products (OIE, International Working Group Team Report 1992).

There is now consensus among the BT experts that there is negligible risk of transmission of BTV through embryos collected from BT positive animals. However, transmission of BTV through semen remains unresolved. The data on transmission of BTV through semen is insufficient to derive final conclusion. There are conflicting reports on the shedding of the BTV in semen from the virimic bulls and rams. The laboratory adopted BTV are more likely to cause foetal infections in pregnant animals and secreted in semen. However, it is not clearly understood why laboratory adopted viruses are more likely to cross the placenta and secreted in semen. In view of the global trade on animal germplasm, it is important that the issue of transmission of BTV through semen is resolved with appropriate and extensive scientific experiment. Clear understanding would help in safe trade on ruminant livestock and their germplasm.

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10

Immune Response

The immune system is an ideal weapon against infectious diseases. It eliminates viruses that invade body and destroys infected cells. The system is very precise and responds only to specific antigens of the foreign invaders. In general the antibodies neutralise pathogens that circulate in body fluids, whereas cytolytic T lymphocytes (CTL) destroy the cells that have been infected by the viruses. Specific immunity against infections is mediated through both humoral and cellular mechanisms. However, the part played by either varies considerably depending upon the infectious agent. Nevertheless, it has been difficult to attribute protective immunity to one or the other component in isolation. The recent understanding gained in the immune response suggests that humoral and cellular responses are intimately connected and often precisely counter balanced by the collaborative cells. It is apparent that depending on the type of immunity required, it may be desirable to induce either strong humoral or cellular response or, in some cases, a combination of both. Despite this, vaccines still seem to be based on their ability to evoke specific antibody response with regard to relevance of protection.

The investigations aimed at delineating the repertoire of immune responses associated with protective immunity and virus clearance in BTV infection have been impeded by inconsistency in experimentally reproducing clinical disease in sheep. Therefore, most investigators currently use development, titre and duration of post challenge viraemia as a primary co-relation (Stott *et al.* 1985). With a view to define immune response to BTV clearly, several *in vitro* and *in vivo* studies have been carried out in the past over three decades. One of most often asked questions about BT is whether or not an animal previously infected with one of the 24 serotypes of BTV is resistant to reinfection with one or more than one serotypes. Is there some degree of protection offered by immune response to a common antigen or is the specificity of the resistance as unique as the antigens that allow *in vitro* typing of the many serotypes of the virus?

Neitz (1948) conducted *in vivo* studies to demonstrate that the inadequate immunity to BTV is because of existence of plurality of antigenically different virus strains. Between these strains there is a variable degree of common or basic immunity, but this is insufficient to protect against infection with heterologous strains. Neitz (1948) also showed that sheep were strongly protected against challenge with homologous strain and

that there was wide variation in virulence among the 10 virus isolates he tested in sheep. Because of this variation, it was not possible to quantitate the degree of immunity established to the heterologous strains of BTV. Luedke and Jochim (1968), in a later study where immunity of several groups of vaccinated sheep was challenged by inoculation of homologous and heterologous virus, clinical response to the heterologous viruses indicated some degree of common immunity similar to that described by Neitz (1948). Groocock *et al.* (1982) immunised sheep with serotype 20 and these were found resistant to challenge with serotype 17. However, this type of immunity was contradicted by Jeggo *et al.* (1983).

Neitz (1948) showed that duration of immunity to homologous virus challenge was 12 months. This period was much longer than 3 months earlier suggested by Du Toit (1929). The fact that different types of viruses appear to be responsible for yearly epizootics of the disease in many parts of the world confirm the initial observations of Neitz (1948).

Immunobiology of bluetongue virus

In late 1940s, it was shown that sheep inoculated with one isolate of BTV or recovered from the infection, were completely protected from the challenge from the same isolate. However, if recovered sheep were inoculated with different isolates, some sheep did not develop protective immunity to that isolate (Neitz 1948). Neitz (1948) further demonstrated that different isolates of the virus differed in virulence. Besides a variation in virulence between BTV serotypes (and strains), sheep vary in the genetic susceptibility to BTV infection. Neitz (1948) reported a variation in susceptibility to a given strain of BTV when different breeds of sheep were inoculated. Merino sheep were less severely affected as compared to Dorsets. Stott *et al.* (1985) reported similar findings using Warhill and Sufolk-cross sheep. They suggested that the differences in disease expression, may, in part be owing to genetic differences in humoral and cellular immune responses of different breeds of sheep. The immune response is important for protection from infection and disease. There is considerable difference of opinion regarding relative roles of humoral and cellular immune responses in both protection and recovery from BTV infection (Ghalib *et al.* 1985a, Jeggo *et al.* 1985). This confusion is partly due to the terms protection and protective immunity, which have been used without proper definitions by various workers. Classically, term protection from infection indicates a mechanism whereby attachment and multiplication of a virus is prevented while protection, on the other hand, implies that only clinical signs of infection are not expressed. Attachment, multiplication, and even spread of the virus may still occur in absence of clinical signs (Mims 1982). The recovery from disease involves the return of an infected, clinically diseased animal to a normal, non-infected condition (Schultz and Grieder 1987).

Besides antibody and cell-mediated immune responses, cytokines have also been found to play an important role in immunity to viral infections. Johnson *et al.* (1978) found that one serotype of BTV was more potent

inducer of interferon than any other viral or non-viral agent studied until that time. They suggested that interferons may play a significant role in non-specific immunity during BTV infection. All the human and animal viruses have been shown to induce interferon synthesis. However, efficiency of induction can vary between different viruses and even different strains of the same virus (Sellers 1964, Lockart 1965). The basic requirement for induction of interferon synthesis appears to be ds - RNA. In ds RNA containing reoviruses, the viral genome itself is an active inducer of interferon (Tylell *et al.* 1967). Huismans (1969) reported that BTV was capable of inducing interferon in mouse embryonic cells as well as mice. He further reported that different strains of BTV differed in their capacity to induce interferon production. In a subsequent study MacLachalan and Thompson (1985) suggested that interferon may be more important in the initial response to BTV infection rather than promoting recovery. Similarly, Jeggo *et al.* (1985) reported that though there is induction of interferon production during an early stage of BTV infection, it does not appear to have major significance in disease prevention or recovery. Other host mechanisms, such as humoral or cellular immunity may play more pivotal role in disease prevention or recovery.

The contribution of antibody versus cellular immunity has not been delineated fully in regard to protection from BTV infection, but the fact that protective immunity and neutralising antibodies are basically serotype specific has suggested that antibody plays an important role in protection. To examine the role of neutralising antibodies in protection from infection, Letchworth and Appleton (1983) passively protected sheep with a BTV neutralising monoclonal antibody.

Humoral immune response

Over the years several *in vitro* tests have been used to detect and quantitate serum antibodies that are produced as a response of host to the virus. The outer capsid is composed of mainly two proteins, VP2 and VP5. VP2 protein has great variability across the serotypes and anti-VP2 antibody levels correlate with neutralising activity *in vitro* and *in vivo* (Huismans *et al.* 1983). Whether VP5 contributes in some way to the neutralising antibody response, is not clearly understood. Although no VP5-specific neutralising monoclonal antibodies have been identified, some studies suggest that this protein plays a role in neutralisation and sero-group identification (Huismans *et al.* 1983, Roy *et al.* 1990). Andrew *et al.* (1985) have shown that VP2 and non-structural protein NS1 contain cytotoxic T-lymphocyte epitopes while VP7 and VP5 are less likely to do so. In the sheep tested to date VP3, VP4, VP6, NS2 and NS3 have not stimulated CTL responses. The viral protein specific humoral immune responses have been characterised in sequential manner by Adkison *et al.* (1988). These workers concluded that BTV infected sheep respond to several structural and non-structural proteins encoded by the viral genome. The heterogeneity of the response is highly dependent on individual animal/breed and species. The role of diverse

antibody populations in protective immunity and/or virus clearance is at present not clearly understood. However, antibodies to different immunodominant domains of VP2 protein alone or in combination with antibodies directed against VP5 protein neutralise BTV *in vitro* as well as *in vivo* in serotype specific manner. Protective role of neutralising antibodies against BTV was suggested by passive protection of mice and sheep against BTV by neutralising monoclonal antibodies (Letchworth and Appleton 1983). These workers injected 6C2A. 4.2 antibodies (which neutralised BTV 17 and precipitates VP2 and VP3 of BTV 17) intravenously into sheep followed by inoculation with BTV 17. These sheep remained free of clinical signs, did not develop viraemia and detectable levels of antibodies by immunodiffusion test. However, low levels of detectable neutralising antibodies were detected. On the other hand the control animals became viraemic and developed high levels of precipitating and neutralising antibodies. This study suggested that antibody directed against single epitope can protect against BTV.

Transfer of antibodies (Jeggo *et al.* 1984a) and T-cells (Jeggo *et al.* 1983) have shown that both effector mechanisms are able to protect BTV infection and or disease. The protection is strongest against homologous serotype (Roy *et al.* 1984) suggesting that virus-neutralising antibody specific for VP2 plays a crucial role, however, it is possible to stimulate heterologous protection (Jeggo and Wardley 1986) indicating that either cross-reactive cell-mediated immunity (Jeggo and Wardley 1982) or antibody to shared epitopes can protect.

Lobato *et al.* (1997) reported that vaccinia recombinantly expressing VP2 proteins did not induce significant immune response against VP2 in rabbits. The immunological response to VP2 is known to be highly confirmation dependent. Huismans *et al.* (1987) were able to induce neutralising immune response against VP2 obtained from BTV 10 after treatment of virions with magnesium chloride but not with denatured VP2 purified from SDS-PAGE gel. However, Marshal and Roy (1990) were able to induce neutralising antibodies in mice inoculated with baculovirus expressed VP2 purified from SDS-PAGE gel. Lobato *et al.* (1997) reported the first direct evidence of neutralising activity in VP5 specific antibody. However, others (Mertens *et al.* 1989, Cowley and Gorman 1989, Huismans *et al.* 1983) published results from BTV reassortants and purified proteins that suggested that VP5 may have a role in neutralisation. In contrast, Marshal and Roy (1990) found no evidence of neutralisation activity in sera from mice inoculated with VP5 produced in baculovirus. Presence of neutralising antibody does not always co-relate perfectly with protection (Luedke and Jochim 1968, Stott *et al.* 1979).

Cell-mediated immune response

There is some evidence to support the hypothesis that BTV specific cytotoxic T-lymphocytes may play a role in protection against BTV infection. Inactivated vaccines can confer protection in absence of detectable level of

neutralising antibodies (Stott *et al*. 1979) and adoptive transfer of BTV-specific CTLs to monozygotic sheep has been shown to confer partial protection (Jeggo *et al*. 1984a, 85). Furthermore, in a laboratory based mouse model, BTV-specific cross-reactive CTLs have been demonstrated (Jeggo and Wardley 1982). These observations were also supported by *in vitro* studies. BTV antigen specific ovine cell lines have been shown to induce cross-reactive stimulation by different BTV serotypes (Ghalib *et al*. 1985a, b, Stott *et al*. 1979). Takamatsu and Jeggo (1989) showed that some CTL lines could reduce BTV replication in autologous skin fibroblast cells. Jones *et al*. (1996) reported that generation of CD^{+8} CTL in two strains of mice (BALB/c and CBA/Ca) against BTV serotype 10. Recombinant vaccinia virus expressing the individual structural and non-structural proteins of BTV was used to infect syngeneic target cells. They observed that both BALB/c ($H\text{-}2^{d}$) and CBA/Ca ($H\text{-}2^{k}$) mice, polyclonal CTL populations, recognised target cells expressing the non-structural proteins. CTL generated against other BTV serotypes also predominantly recognised the non-structural proteins. However, the extent of cross-reactivity was dependent on the H-2 background of the animals immunised. No CTL cross-reactive to panel of molecularly cloned recombinants in the $H\text{-}2^{d}$ haplotype were generated. The outer capsid proteins VP2 and VP5 which vary significantly between serotypes were not recognised by heterotypic CTLs. Using the murine model Jones *et al*. (1996) determined which BTV proteins are the major targets of the CTL response. They observed that the non-structural proteins which had elicited the strongest homotypic CTL response in both haplotypes of mice, also dominated the heterotypic CTL response to individual recombinant protein in the $H\text{-}2^{k}$ mice. However, NS2 did not cross-react with serotype 2, suggesting that NS2 allele-specific peptide motifs were not shared throughout the BTV serotypes. The target cells infected with recombinant vaccinia virus synthesise the most variable proteins VP2 and VP5. Janardhana *et al*. (1999) subsequently, reported that analysis of virus/CTL interactions at molecular level and localisation of CTL epitopes within BTV non-structural proteins would add in design of a BTV multi-component vaccine which could theoretically boost CTL memory. Studies in sheep are required to determine if shared epitopes are selected in different MHC background (Jones *et al*. 1996).

Transfer of BTV primed lymphocyte has shown that CTL are able to protect sheep from BTV disease (Jeggo *et at*. 1985). Lobato *et al*. (1997) found no relation of CTL activity and protection. They suggested that virus neutralising antibodies rather than CTL play the dominant role in protection.

Some investigators have suggested that cell-mediated immune response is responsible for protection from BTV re-infection and sheep produce such a response on immunisation with an inactivated vaccine (Stott *et al*. 1979). Cytotoxic T cells have the ability to destroy virus-infected cells early in infection and thus prevent viral spread to other cells/tissues.

The studies conducted during the past over fifty years have mainly focused on the mechanism and kinetics of the immune response in sheep

against the whole virus either after vaccination with inactivated/attenuated vaccines or as result of natural infection with BTV. However, in the past over a decade novel approaches have been used to develop effective recombinant immunogens and new adjuvants. The combination of recombinant antigens, adjuvants and cytokines has exhibited potential for better and long-lasting immune response. Molecular mechanisms of cellular as well as humoral immune responses in different ruminant species particularly sheep and cattle are still required to be worked out. It is not precisely known how the virus and the virus specific neutralizing antibodies co-exist? Similarly mechanism of longevity of immune response is poorly understood. Better understanding of the immune response would help in designing better vaccines.

Detailed immunological studies with regard to Indian breeds of sheep are lacking. Keeping in view the economic importance of BT, an All India Network Project has been initiated in the country with 9 centers. One of the objectives of the network project is to develop an inactivated vaccine. Some preliminary work has been initiated to achieve this objective at the Indian Veterinary Research Institute, Izatnagar, India.

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11

Diagnostics

Prompt detection of infectious agents in animals as well as ecosystem is imperative for effective control and management of infectious diseases. To control any disease causing agents, it is necessary to first diagnose it. For centuries diagnosticians have tried to identify individual infectious agents by their clinical signs and symptoms produced in the susceptible host. But it is not always possible to identify or differentiate the disease on the basis of its signs and symptoms and thus requires other diagnostic procedures to clearly identify and confirm the disease aetiology which is desirable or, indeed essential. Bluetongue virus has an architecturally complex structure. It has an ability to attach and replicate in a variety of vertebrate and invertebrate cells. The diagnosis of BT in sheep is uncomplicated for an experienced clinician. However, the disease in cattle can be confused with infectious bovine rhinotracheitis, malignant catarrhal fever and bovine viral diarrhoea/mucosal disease. The diagnosis on the basis of apparent clinical signs and symptoms exhibited by the BT affected animals is not a problem, whereas field diagnosis of sub-clinical and inapparent infection of BTV presents a difficult situation. Therefore, different laboratory methods are required to detect the virus, its antigens, antibodies, and the viral nucleic acid. During last couple of decades advances in molecular biology, immunology and virology have provided unprecedented opportunities to develop innovative biotechnological approaches using molecular techniques such as recombinant-DNA, hybridoma, *in vitro* enzymatic amplification of nucleic acid, hybridisation technologies for producing more effective, economical, thermostable, specific and sensitive diagnostic reagents.

Coventional diagnostic approaches

Over the past few decades, diagnostic virology has proliferated in a big way and now one has an access to wide variety of tests. Laboratory diagnosis of any infectious disease depends upon light microscopy, *in vitro* immunological reactions and tissue culture.

Bluetongue virus was first diagnosed as epizootic catarrh (Hutcheon 1881) based on clinical signs. Spreul (1905) named it bluetongue owing to appearance of cyanosed tongue. The field diagnosis of BT is usually made by clinical signs characterised by pyrexia, swelling of muzzle, oral lesions, coronitis, stiffness of limbs and in some cases oedema of head and neck. The clinical BT has been dealt elsewhere in some other chapter in this book.

However, field diagnosis of sub clinical and inapparent infection is difficult because of similarity in symptoms and signs with other diseases like sheep-pox, foot-and-mouth disease, contagious ecthyma, ulcerative dermatitis, photosensitisation and pneumonia. Therefore, various diagnostic methods are required to detect BTV. Tests for the specific diagnosis of BTV infection are of generally three types, viz. (i) virus isolation in susceptible chicken embryo, animal or tissue culture; (ii) those that demonstrate the presence of viral antibodies, viral antigen; and (iii) those that demonstrate the presence of viral nucleic acid. The conventional diagnosis usually involves isolation of the virus or the detection of specific antibodies in the serum of convalescent animals.

Virus isolation

Despite the explosion of new technologies for same day diagnosis of viral diseases by demonstration of virus, viral antigen or viral nucleic acid in specimens taken directly from the animal, it is still true to say that few of them achieve quite the sensitivity of virus isolation in embryonated chicken eggs or cell culture. Theoretically at least, a single viable virus present in a specimen can be grown in cultured cells, thus expanding it many fold to produce enough viral antigen. Virus isolation remains the gold standard and must be compared with other methods available. Growth of viruses was restricted to animal inoculation. Initially sheep inoculation was used as the diagnostic method for BTV. Subsequently developing chicken embryo was used for isolation of the virus. Rous and Murphy (1911) used the embryonated chicken eggs (ECE) for virus propagation. Andrews and Rivers (1920) first recognised the CPE in tissue culture. Gey and Enders (1950) paved the way for the application of cell cultures in the routine diagnosis of most viral diseases. Culture is the only method of producing a supply of live virus. Moreover large quantities of virus must be grown up in culture cells to produce diagnostic antigens.

The isolation of virus from the clinical specimens is most reliable classical way of confirmatory diagnosis of BTV. There is no optimal protocol for the isolation of the virus. The chance of isolating the BTV depends critically on the attention given by the attending clinician or veterinarian to the collection of the specimen. Clearly such specimens must be taken from the right place at the right time. The right time is as soon as possible after the animal first develops clinical signs evidenced by high rise of temperature because the virus is present in maximum number in blood circulation at about this time then falls down. Inoculations of the suspected clinical material in susceptible sheep, suckling mice, ECE and a variety of vertebrate and non-vertebrate insect cell lines have been used for isolation of the virus. It was only in 1940 that BTV was propagated in ECE and later in 1956 in various cell lines (Haig *et al.* 1956). Antigen capture enzyme linked immunosorbent assay has been evaluated for rapid screening of embryonated chicken eggs for the presence of BTV (Hosseini *et al.* 1998). Since the virus is transmitted by insect vector, susceptible sheep inoculation is normally not

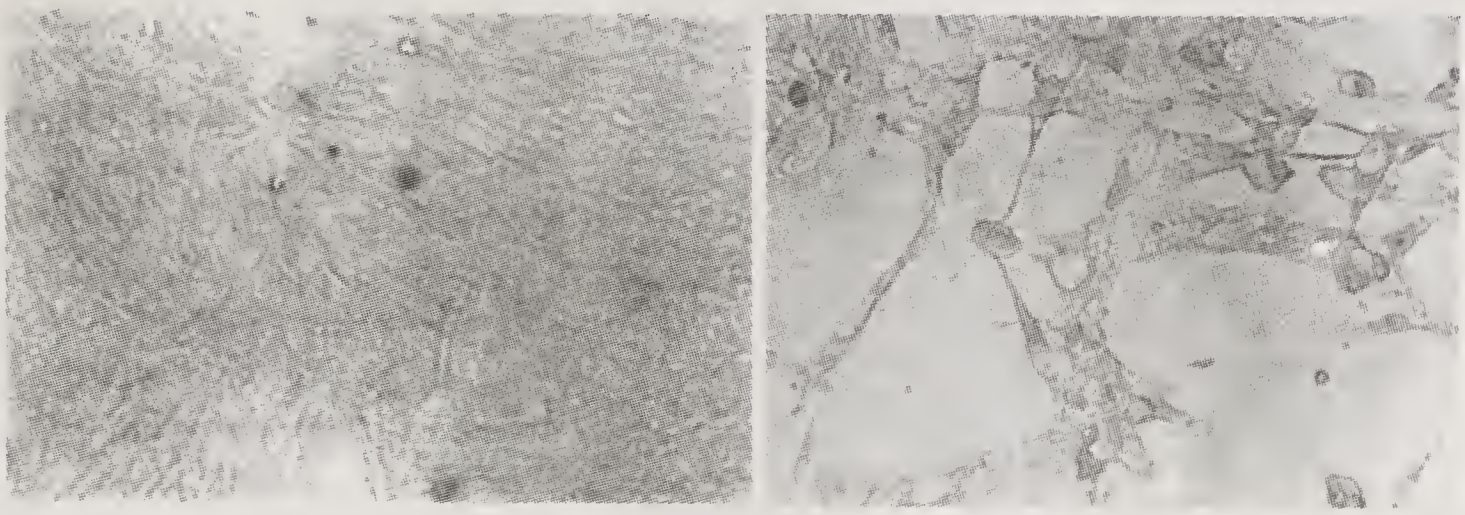


Fig. 31. Isolation of bluetongue virus in BHK-21 cell line. A: Normal uninfected BHK- 21 cell monolayer. B: BTV infected BHK 21 cells showing cytopathic effect characterized by rounding and degeneration of the infected

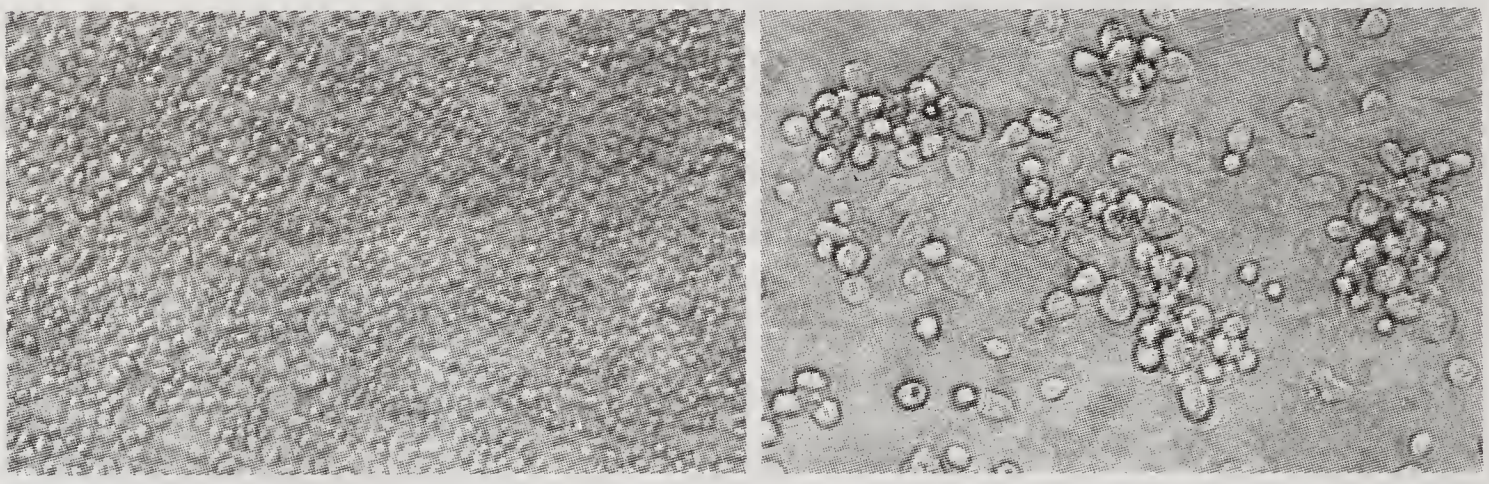
used for the diagnostic purposes because it requires costly midge-proof housing system and safety procedures. Inoculation of 9-10-day old ECE is a common method for primary isolation of the virus. However, inoculation of mammalian-cell line BHK-21 is the most versatile system used for the isolation of BTV (Fig. 31. A, B). In the recent years cell lines of insect origin have been found better than the mammalian cell lines for isolation of the virus. Jochim (1985) reported that the sensitivity of intravenous inoculation of ECE and sheep inoculation for BTV isolation was similar and cell culture was slightly less sensitive while suckling mice inoculation was least sensitive. However, no one system is the best and chances of the virus isolation are better if a number of isolation systems are used simultaneously. Generally for primary isolation of the virus, initial inoculation of ECE followed by consecutive passages in the mammalian cell culture or insect cell line (Clavijo *et al.* 2000) is practiced. ECE after BTV infection show stunting growth with oedema and haemorrhages all over the body (Fig. 32).

In spite of the high sensitivity of sheep and ECE inoculation for isolation of BTV, *in vitro* cell culture systems have been effective for isolation. Wechsler and McHolland (1988) evaluated susceptibility of various cell lines for isolation of BTV and concluded that calf pulmonary artery endothelium (CPAE) cells were most sensitive (Table 25). However, other susceptible cell lines include Vero, BHK-21 and C6/36 (Fig. 33 A, B).

Fig. 32. Chicken embryo inoculated with BTV, showing stunting of growth and generalized haemorrhages.

Table 25. Different cell lines used for BTV isolation

Cell line	Cytopathic effects
Big horn sheep foetal tongue explants (BHFTE)	Cell death
Baby hamster kidney BHK21 clone 13	Cell death
Bovine turbinate (bt)	Cell death
Aedes albopictus (C6/36)	Vacuolation and clumping of cells
Calf pulmonary artery endothelium (CPAE)	Cell death
Foetal ovine lung fibroblast (CSL 503)	Cell death
Culicoides variipennis (CuVa)	Cell death
Monkey kidney (CV-1P)	Cell death
Mouse fibroblast (L929)	Cell death
Rhesus monkey kidney (LLC-MK2)	Cell death
Rabbit kidney (LLC-RK1)	Cell death
Madin Darby bovine kidney (MDBK)	Cell death
Equine dermis (NBL-6)	Cell death
Super vero porcine stable (SVP)	Cell death
African green monkey kidney (Vero)	Cell death



A B

Fig. 33. A & B. Insect cell line (C6/36) infected with BTV. A: C6/36 normal uninfected control. B: C6/36 cell line infected with BTV showing rounding and clumping of the infected cells.

Detection of the virus and its antigens

The growth of viruses is generally slower than direct detection assays. Over the past few decades antigen detection has become an essential part of laboratory diagnosis. Since the virus isolation is very time consuming and laborious method, direct detection of the virus and its antigens in clinical specimens provides a rapid means of diagnosis. The commonly used assays include immunoperoxidase, immunofluorescence and enzyme immunoassays. Immunoperoxidase test has been used for detection of BTV antigens in the specimens obtained from infected chicken embryo, cell culture, mononuclear cells (Garg and Prasad 1996). Avidin-biotin immunoperoxidase procedure with more specificity has also been used to detect BTV antigens in the tissues of BTV infected calves (MacLachlan *et al.* 1990). The suggestion was made that the method could replace the fluorescent antibody test (Fig. 34), which generally lacks specificity, but the immunoperoxidase technique

has not found wide application. Direct and indirect immunofluorescence tests were used for detection of BTV antigens (Garg *et al.* 1994). (Fig. 25).

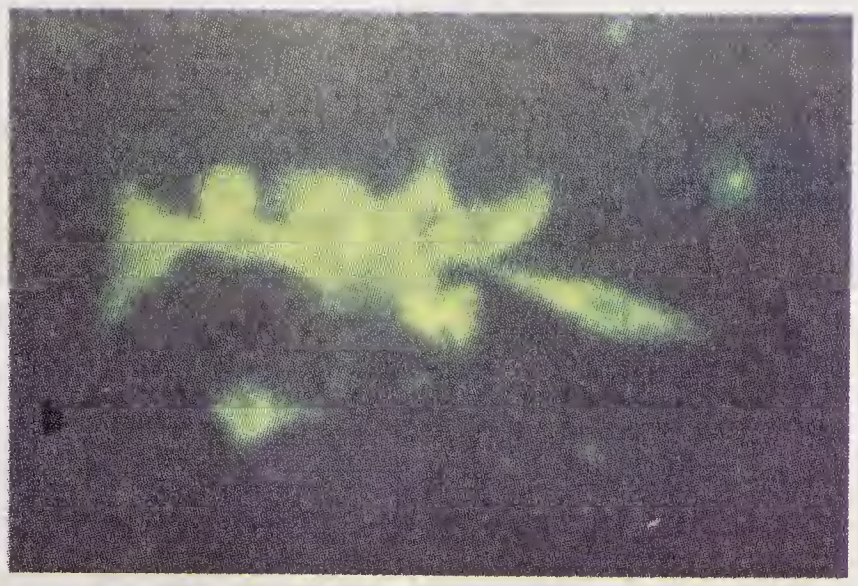


Fig. 34. BHK-21 cell line infected with BTV and stained with FITC. The infected cells are showing extensive green fluorescence.

Electron microscopy is perhaps the most obvious method of virus identification by direct visualisation of the virus itself. This has also been useful in tracking the BTV, BTV-like particles and BTV antigens in infected tissue culture cells (Fig. 35) and other type of cells infected *in vivo*. It is also used for detection of BTV in infected blood samples of animals (Prasad *et al.* 1993).

Low sensitivity is the biggest limitation of this assay as a diagnostic tool. A skilled microscopist is needed and it uses an expensive machine to scan the grid adequately and detect virus when the specimen contains fewer than 10^7 virions per milliliter. Specimens are stained negatively with phosphotungstate, or sometimes uranyl acetate and then scanned by electron microscopy. Modification in the basic EM was made by using immunogold electron microscopy by adding specific antibodies labeled with gold. Solid phase immunogold EM techniques have been developed in which virus specific antibody is first bound to the plastic supporting filter on the copper grid. Adkinson *et al.* (1988) studied the temporal development of humoral immune responses in sheep to natural BTV infection using Western immunoblotting. The procedure was superior to serum neutralisation and agar gel immunodiffusion tests in identifying post exposure to the virus. The immunoblotting procedure appeared to be group specific when assaying ruminant sera. Sera from BTV infected sheep did not cross react with EHDV proteins and vice-versa.

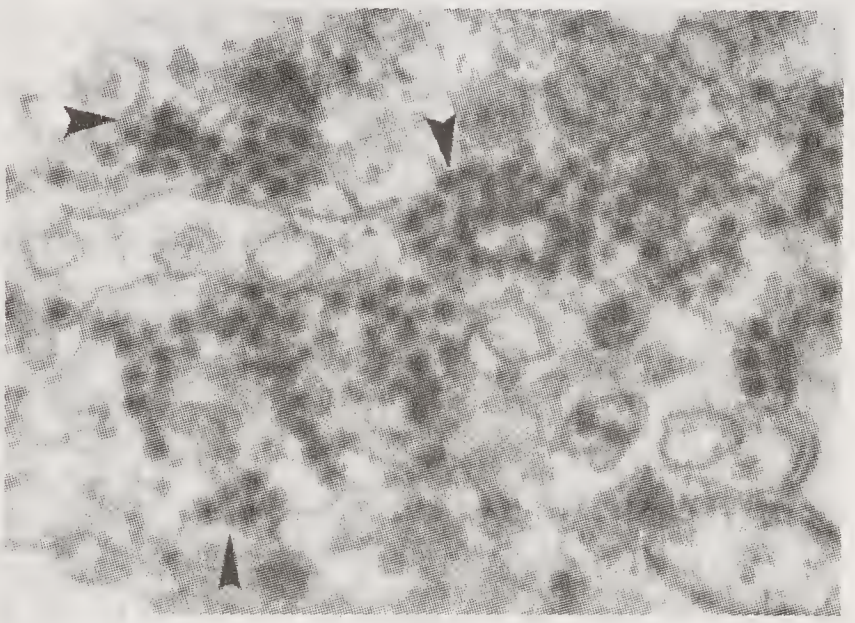


Fig. 35. BHK -21 cells infected with BTV, exhibiting several virus particles in the cytoplasm of the cells by transmission electron microscopy (62, 000 X).

Detection of anti-viral antibodies

The serological assays of proven sensitivity and specificity are routinely

used in epidemiological surveys, disease control programs and even regional eradication programs. Serology based on host's immune response to viral infection has more impact than other diagnostic assays in use. Serological assays depend on the detection of antibodies in the paired sera samples from an acute and convalescent serum of the same animal. The interpretation and value of a particular serological test is critically dependent on an understanding of two key parameters, viz. sensitivity and specificity. The sensitivity of a given test is expressed as a percentage of animals with disease/infection in question that are identified as positive by that test, divided by the total number that have the disease. In contrast specificity of a test is a measure of the percentage of those without the disease/infection who yield a negative result. The sensitivity of a given immunoassay is also a measure of its ability to detect small amounts of antibody.

Presumptive diagnosis of BTV infection has been made by serological tests rather than by isolation and identification of virus. Serological tests have played an important role in determining the distribution of BTV infections and these are the primary methods used to certify animals for export. Detection of antibodies to BTVs is based on group reactive tests and the serotype specific neutralisation tests. A number of traditional serological methods such as agar gel immunodiffusion (AGID) test, counter current immunoelectrophoresis, complement fixation, fluorescent antibody tests etc. have been used. The serological tests used to detect BTV antibodies are of two types, viz. group specific and serotype specific. Serum neutralization test (SNT) and haemagglutination inhibition test are serotype specific. Of the two, SNT is the most widely used test. SNT requires cell culture facilities, thus haemagglutination inhibition was considered as an alternate test. As outer capsid protein VP2 has a direct role in determination of serotype specificity, alternative tests using serotype specific reagents-like antigens or monoclonal antibodies (MAb) could be developed. However, this would require development of MAb to all the 24 known serotypes.

Tests devised for the detection of group specific antibodies include agar gel immuno diffusion test (Jochim and Chow 1969), complement fixation test, dot-immunobinding assay (DIA), enzyme-linked immunosorbent assay (ELISA) and competitive-ELISA (c-ELISA). All these tests are based on the detection of major inner core group specific protein VP7 that is highly conserved and reacts with antisera against all the 24 known serotypes of BTV. Complement fixation test suffers from many problems, especially anti-complementarity of sheep sera and lack of reproducibility. However, addition of normal bovine serum to the test enhanced the level of BTV specific antibodies. The AGID test replaced the complement fixation test as it is easy to perform and is reproducible. The AGID test for BT was first described by Jochim and Chow (1969) and has been used in a variety of modifications to detect antibodies to BTV. It remains most widely used test in spite of the fact that it lacks sensitivity and shows some degree of cross-reactions with related Orbiviruses (Della-Porta *et al.* 1983). The test is easy to perform and is inexpensive therefore; it is often preferred in surveying

animals for BTV infections. The reports that AGID test failed to detect antibodies in a significant number of animals, which were positive for BTV, and the problems of cross-reactivity suggested that the most likely serological test to replace AGID is ELISA. Two types of ELISA have been used to detect BTV infections. In the indirect ELISA the viral antigen is coated on polystyrene plates and reacted with test serum or serum containing antibodies. In authors' laboratory, dot-immunobinding assay (Fig. 36) was found quite sensitive as compared to AGPT and SNT (Chander *et al.* 1991).

A rapid IgM class specific antibody assay based diagnosis of BTV can be made on the basis of a single acute phase serum by demonstrating virus specific antibody of IgM class. IgM antibodies appear early after infection and drops within 3 months, and are usually indicative of recent infection. The most common method used is the IgM antibody capture assay, in which the viral antigen is bound on a solid phase matrix, say a microtitre well. The test serum is allowed to react with this antigen and then specific IgM antibodies capture by the antigen are detected with labeled anti -IgM antibody matched to the species from which samples were obtained. Zhou *et al.* (2001) developed and evaluated an IgM capture ELISA for detection of recent infection with BTV in cattle. The test is based on the use of biotinylated capture anti-bovine IgM antibodies bound to a streptavidine coated ELISA plate. The captured IgM antibodies were detected by application of BTV VP7 antigen and a VP7 antigen specific MAb. This assay was capable of detecting all 24 serotypes of BTV at 10 days PI, whereas cELISA was unable to detect. IgM antibody ELISA is sensitive and can be applied for the detection of recent infection of BTV in cattle.

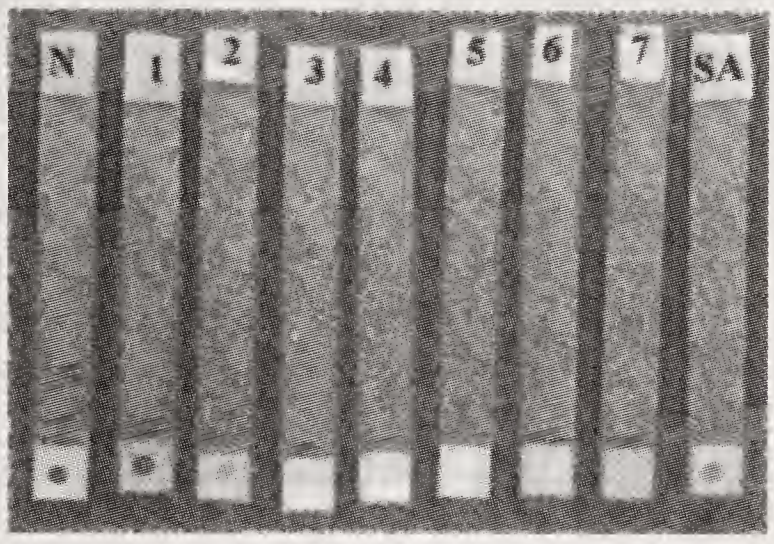


Fig. 36. Dot immunobinding assay showing dots on the dipstick with BTV positive sera

It is now clear that the group specific antigens of BTV may share minor epitopes with those of other *Orbiviruses*, thus immuno diffusion and ELISA may detect cross-reactive antibodies to viruses belonging to serogroups other than BTV. Therefore, blocking-ELISA was developed by Anderson (1984) using MAb for detection of group specific antibodies to BTV. In this test immobilised antigen is reacted with a test serum and then with a group specific murine monoclonal antibody. Antibody to BTV if present in the test serum, blocks the antigen, preventing reaction with monoclonal antibody in the last step of the test. Sera containing EHD virus do not react in the test. Antibodies to cellular proteins, which can complicate interpretation of immunodiffusion tests and indirect ELISA tests, do not interfere in blocking-ELISA. This was more specific and sensitive diagnostic

test for BTV. The results of Anderson's blocking ELISA were confirmed by using MAb and a competitive-ELISA format (Afshar *et al.* 1987). Blocking-ELISA and c-ELISA were found equally sensitive and specific showing no cross-reaction with epizootic EHDV of deer and superior to indirect-ELISA. Jeggo *et al.* (1992) defined c-ELISA protocol and set of reagents that could provide basis for an internationally accepted standard.

Biotechnological diagnostic approaches

Several conventional methods of diagnosis are still in use, most of them are too slow to have any direct influence on clinical management of a particular case, providing results after several days. Modern diagnostic procedures using biotechnological approaches are gradually replacing other conventional diagnostic methodologies because of advantages of simplicity, automation, sensitivity, specificity and flexibility.

A major thrust of the development in modern diagnostic virology has been toward rapid methods that provide a definitive answer in less than 24 hours or even during the course of the initial examination of the animal. The best of these methods fulfill five prerequisites: speed, simplicity, sensitivity, specificity and low cost.

Recombinant antigen and monoclonal antibodies based assays

Over past two decades, significant advances have been made in understanding of the structure-function relationships of the BTV genes and gene products. Availability of cDNA clones and baculovirus expression vectors led to production of a number of diagnostic reagents. The development of hybridoma technology and genetic engineering paved the way for production of well-defined and highly specific non-cross reacting diagnostic MAbs and a variety of recombinant diagnostic antigens. The BTV group specific antigen VP7 was expressed in yeast and *S. frugiperda* insect cells. The use of expressed VP7 antigen as a diagnostic reagent in blocking-ELISA and c-ELISA for the detection of BTV antibodies in sera collected from infected animals were reported by Eaton *et al.* (1990) and Martyn *et al.* (1990). Of the two, yeast-expressed VP7 was widely evaluated for c-ELISA. Naresh and Prasad (1995) also used c-ELISA based on yeast-expressed VP7 and non-cross reacting MAb for detection of BTV antibodies in domestic ruminants. Naresh *et al.* (1996) also observed that recombinant VP7 antigen produced by yeast and baculovirus expression systems was as good as the conventionally cell-culture produced group specific antigen in dot-immunobinding assay. The advantages of recombinant antigens over conventional cell culture derived antigens will make them widely utilized antigens in coming years. The cost associated with the cultivation of virus in cell culture is more as compared to cost of production of recombinant antigens. The genetically engineered antigen is likely to be more consistent and uniform than cell culture derived. Recombinant antigens are non-infectious and can be used worldwide without the fear of infecting animals of BT free regions.

Detection of viral nucleic acid

Over the past one decade there has been an explosion of information on the BTV genome and this will serve as a basis for next generation of nucleic acid based diagnostic techniques that would be more rapid and sensitive. These techniques rely not on the isolation and identification of a live replicating virus, but on the detection of highly specific molecular subunits of the virus in infected tissues and clinical specimens. These subunits could be either gene sequences specific for BTV or certain serotypes. Obviously, direct detection of these molecular subunits from infected cells or insect vector is more efficient diagnostic approach. For the detection of virus specific nucleic acids, RNA electrophoresis, cDNA/RNA blot hybridisation and polymerase chain reaction to amplify the small amount of nucleic acid *in vitro* have been developed using the knowledge of BTV genome and recombinant DNA technology.

RNA electropherotyping: Due to segmented nature of BTV genome it is possible to separate the genomic segments using RNA electrophoresis. It has been demonstrated that all the 10 segments of ds viral RNA can be separated by polyacrylamide gel electrophoresis (PAGE). Different serotypes of the virus have the distinct pattern of ds-RNA segments migration on PAGE. Hence, the RNA-PAGE can be used as a preliminary diagnostic tool to identify the BTV isolates from field samples (Sugiyama *et al.* 1981). In the authors laboratory, it was experienced that while attempting isolation of the virus either from blood of the suspected animals or insect vector, the virus is not detectable by DIA at early passage levels. The comparative evaluation of DIA and RNA-PAGE suggests that RNA-PAGE and DIA have comparable sensitivity and both tests can detect a minimum virus titre of 10^5 $TCID_{50}$/ml. However, RNA-PAGE has advantage over dot immunobinding assays in that it identifies the RNA segment migration pattern of the virus; hence it is less likely to yield false positive results as sometimes may be the case in enzyme immunoassays. More over, RNA-PAGE also helps in detecting the different genotypes of the same serotype or different serotypes while group specific immunological assays cannot give any idea about the difference in the viral genomic RNA diversity. The RNA-PAGE technique in addition to comparable sensitivity with enzyme immunoassays, also permits assessment of genetic relationships and provides a useful tool for the analysis of RNA segment reassortments in crosses between isolates of BTV. Migration pattern of genomic RNA segments based on molecular weight and presence of additional bands in the PAGE technique also provides an opportunity to identify mixed infections of the animal host and insect vector by more than one serotype or reassortments which is not possible by enzyme immunoassays. A preliminary study has indicated that two isolates of the same serotype of BTV from different geographical locations have different viral RNA genome profile on RNA-PAGE (Fig. 37) (Prasad *et al.* 1998).

The RNA- PAGE method is reasonably rapid (less than 12 hours) and can be performed with small volumes (0.1-1ml) of BTV infected cell culture

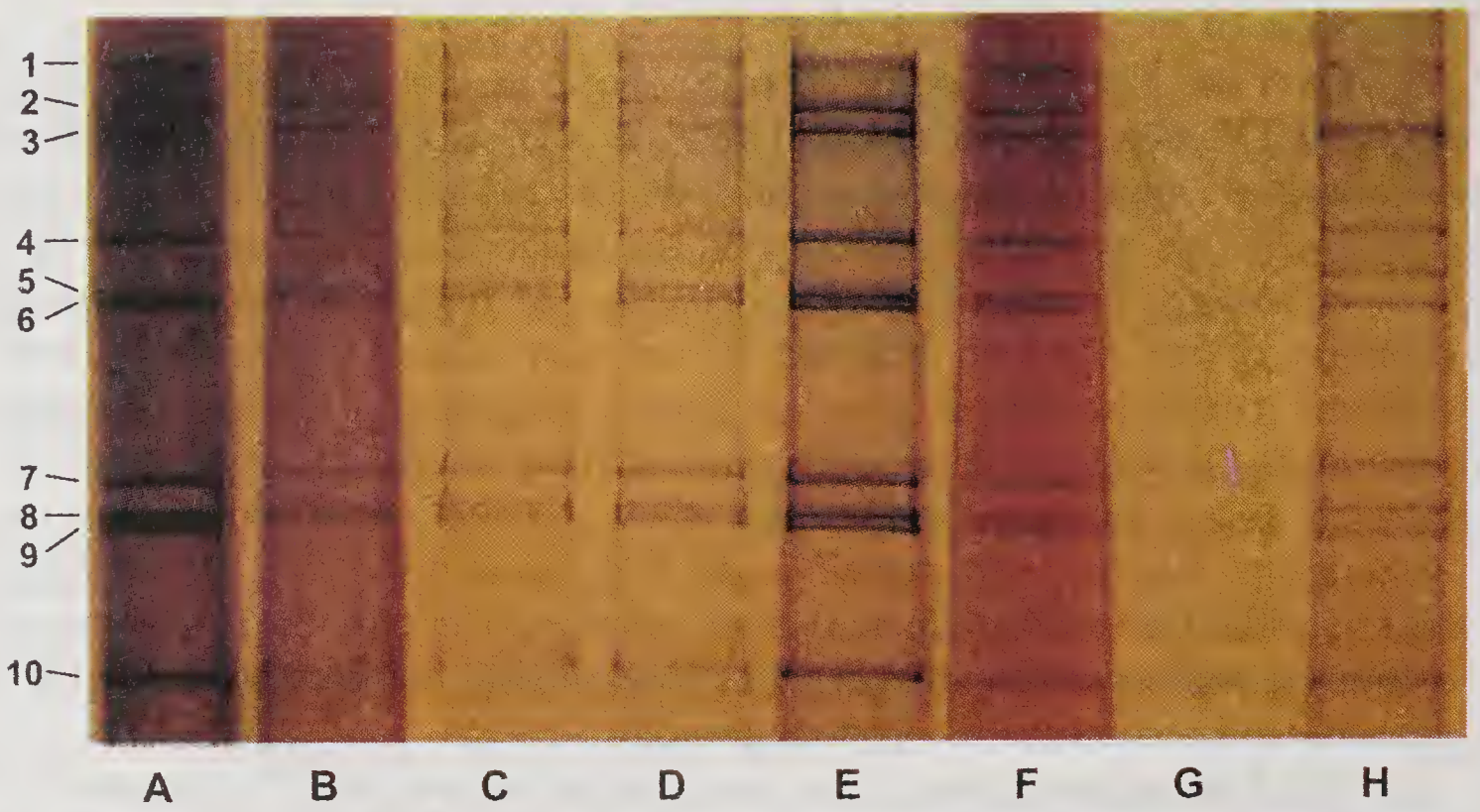


Fig. 37. Polyacrylamide gel electrophoresis showing migration patterns of genome segments of different isolates of BTV.

debris (Squire *et al.* 1983) or infected clinical material including blood and insect vector.

Nucleic acid hybridization

The sensitivity and versatility of nucleic acid hybridisation (NA) techniques have been expanded rapidly such that probing for the viral genome has overtaken probing for antigen as the diagnostic method of choice. The NA hybridization technique offers several features including high sensitivity, specificity and rapid screening of target sequences. The detection of specific viral NA by hybridisation using labeled viral DNA/RNA probes to detect NA has been widely used for rapid diagnosis although the methods have been superseded in many instances by the polymerase chain reaction. The use of recombinant cDNA probes make standardization of test regulation easier in comparison to serological assays which are based on polyclonal reagents in spite of the minor drawbacks-like special processing of samples required to make target nucleic acid available for hybridization in purified form and optimisation of assay conditions. In principal NA hybridisation requires the hybridisation of ss DNA or RNA or cDNA probe by hydrogen-bonded base pairing to another ss of DNA or RNA of complementary base sequence. Thus two strands of the target molecule are first separated by heating and allowed to hybridize with a labeled single stranded probe. Different methods have been used for labeling of nucleic acids to be used as probes with radioactive and non-radioactive labels.

Traditionally radioactive isotopes such as ^{32}P and ^{35}S were used to label NA sequences or probes for hybridisation tests, with signals being read by autoradiography. The trend is now towards non-radioactive probes. For radio-labeled probes the radio-isotope is incorporated at the ends of NA fragments and the choice of method depends upon amount of NA available, its size in

base pairs, type of NA (RNA or DNA) and whether it is single stranded or double stranded. The process of nucleic acid hybridization, involves the interaction between labeled cDNA/oligo probes and complementary target RNA or DNA to form a DNA-DNA or DNA-RNA hybrids. Generally such probes have ends, which can be labeled. The 5'end of NA can be labeled with ^{32}P using T4 polynucleotide kinase which catalyses the transfer of g- phosphate group from ATP to 5' -OH terminus of DNA molecule. Using this method synthetic oligonucleotide or ds DNA fragments after dephosphorylation can be labeled. Normally 3' end of the DNA fragment can be labeled by incorporation of a ^{32}P label using terminal deoxy nucleotidyle transferase (Tdt) which catalyses the repetitive edition of mono nucleotides from deoxynucleotide triphosphates (dNTPs) to the 3'-OH terminus of single stranded or ds DNA. Thus this enzyme provides a unique method for labeling probes with ^{32}P for use in hybridization assays. The DNA probes prepared by nick translation can be used for wide variety of hybridization technique like gel blot, colony and plaque lifts. Now-a-days random labeling system using random hexamers is commonly used to prime DNA synthesis *in vitro* from any linear ds DNA template to a high specific activity.

Greater emphasis is now being given to the development of non radioactive label probes as these are safer, cause less health hazards, economical and reduce cost of storage, no problem of self-life and their disposal. Non-radioactive labels can be incorporated by random prime labeling, nick translation, using terminal transferase and PCR amplification. Some of these non-radioactive labels in common use in BTV diagnosis are as biotin or digoxigenin, which act indirectly by binding to another labeled compound which ultimately emits the signal.

Two basic methods for detecting BTV nucleic acid using hybridization technique were reported extensively; first one is based on the identification of viral nucleic acid sequences bound to solid matrix like nitro cellulose membrane (NCM) or nylon paper. Now-a-days commercially available dot blot, multifold vacuum filtration units allow multiple samples to be blotted directly, quickly and uniformly on to the solid matrix. Dot blot hybridisation assay is becoming more popular because of its simple format. In this nucleic acid (viral RNA) extracted from virus or infected cells is denatured and then spotted directly onto a charged nylon or NCM to which it binds tightly on UV cross linking. The single stranded DNA or RNA probe is then hybridised to the target NA on the membrane and unbound probe is washed off. The signals after specific immunological protocols are observed after adding the specific substrate. By choosing mRNA as a probe, sensitivity can be improved many folds. Second method involves detection of nucleic acid within specially prepared, intact cells or tissues (*in situ* hybridization).

For the detection of the virus specific nucleic acids, cDNA/RNA blot and *in situ* hybridization techniques have been developed using the knowledge of BTV genome and recombinant-DNA technology. A number of nucleic acid probes have been evaluated in different laboratories for detection of the viral genetic sequences in tissues and other body fluids (Roy

et al. 1985). The asymmetric single stranded RNA probes were used to identify the relative amounts of positive and negative sense NA strands for studying the kinetics of viral nucleic acid products. These probes are used to distinguish viral mRNA and viral ds RNA components (Dangler *et al.* 1988). The problems of using a single genome segment as most suitable group specific probe is compounded by the fact that there is significant variation in conserved genes. Although VP7 has group reactive epitopes and is the protein utilised in the group specific BT serodiagnostics, yet gene encoding VP7 shows greater sequence variation within serotypes and can not be recommended as target for group specific probe. The BTV genome segments corresponding to VP3 (segment 3) and NS1 (segment 6) showed greater sequence conservation and are being evaluated as group specific probes (Wilson 1994). A DIG labeled cDNA probe prepared by labeling the semi-nested PCR product of NS1 gene has been evaluated for detection of the virus in spiked blood and semen samples in authors' laboratory. Results suggest that DNA probes could be used for detection of BTV infection in blood and semen of the carrier animals (Fig. 38). Brown *et al*. (1996) determined the distribution of BTV in tissues of experimentally infected pregnant dogs using the *in situ* hybridisation assay. In the assay digoxigenin labeled probe corresponding to the NS1 gene was used.

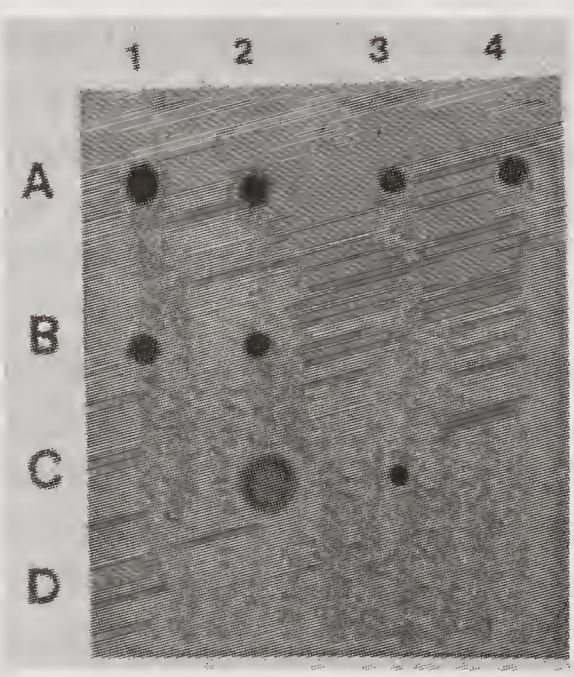


Fig. 38. RNA-DNA hybridization using DIG labeled DNA probe. Dark violet dots indicate positive hybridization signals

Variations in DNA hybridization protocols were developed to suit to the specific requirements of different clinical specimens and the viruses. *In situ* hybridization method is particularly very suitable for detection of the virus in tissues and cells. The advantage of this method is that it allows identification of the viral RNA/ DNA sequences within morphologically distinct cells in the infected tissue sections. In this procedure, the tissue sections fixed in methanol, acetone, formaldehyde and glutaraldehyde can be used for detection of the desired nucleic acid. Frozen sections can also be used directly in this method.

Polymerase chain reaction

Polymerase chain reaction (PCR), discovered by Karry B Mullis (1983), revolutionised the whole field of molecular biology (Saiki *et al.* 1986). Mullis for this discovery received the Noble prize. PCR technology has its wide spread applications in the field of genetics, forensic medicine, parentage determination, and evolutionary biology and has attained a paramount importance in diagnosis of infectious and non-infectious diseases due to its speed, sensitivity and specificity. The immense potential of the PCR to specifically amplify minimal amounts of target DNA was particularly

appealing for the diagnosis of viral diseases. Although less spectacular in veterinary science in comparison with human medicine, there has been a rapid expansion in the laboratory diagnosis based on PCR. PCR is being widely exploited to detect viral nucleic acids in clinical specimens as a very rapid alternative to other virus detection methods. It is now possible using the PCR to provide a comprehensive diagnostic service within 4-5 hours from specimen submission. A traditional method of viral diagnosis generally involves cumbersome method of isolation of the virus in mammalian or insect cell cultures followed by confirmatory tests. The time required for identification of viral agent might take 7-10 days. The success of growth of a particular virus is also dependent on the quality of the sample submitted and to the time taken to reach to the diagnostic laboratory, as successful isolation of the virus requires the presence of viable organism in the clinical sample. In contrast, use of PCR offers the possibility of same day diagnostic results and is much less dependent on the conditions in which the sample arrives. DNA has even been amplified using PCR from mammoths extinct for thousands of years (Binns 1993).

In principal PCR is an *in vitro* method for enzymatic syntheses of specific DNA sequences using two oligonuclotide primers that hybridises to opposite strands and flanks the region of interest in the target DNA. The primer extension products synthesised in one cycle serve as templates in the next, hence the number of target DNA copies approximately doubles every cycle and after 20 cycles a million-fold amplification is obtained. For PCR-based diagnosis, DNA sequence of the target viral pathogen must be available. The basic procedure requires: two short DNA primers based on the complementary sequences of the target viral genome and these primers bind to the opposite strands of ds DNA or cDNA (in RNA viruses), four bases of dNTPs for DNA synthesis and a thermostable DNA polymerase (Taq). The Taq polymerase is stable in high temperatures needed to denature the DNA between the cycles, which circumvents having the need to add fresh enzyme after each denaturation stage. The mixture is briefly heated at 94°C to separate DNA strands. The mixture is rapidly cooled to a temperature at which the primers anneal to their target sequences and then the DNA polymerase synthesises new DNA between 2 primers by adding dNTPs. Heating and cooling is repeated for about 30 cycles with theoretical doubling of the target sequence with each cycle. The whole process is completed within a couple of hours. During this time the target sequence is amplified several million fold to make the detection of amplified target a simple task (Binns 1993).

In the specimens where small number of viral particles is suspected, PCR could be used to amplify the viral nucleic acid. The evaluation of PCR for detection of BTV in cell cultures and in clinical specimens including blood and semen has been done (Dangler *et al.* 1990). Wade Evans *et al.* (1990) have successfully used PCR to detect BTV in blood samples of cattle experimentally infected with BTV. PCR reproducibility allowed detection of as few as 6 molecules of ds RNA, after 60 reaction cycles.

Moreover, use of PCR for detection of BTV eliminates detection of closely related EHDV. PCR could be used for detection of BTV in semen and embryos also. Various PCR protocols have been developed in the last decade. Recenty VP7 (Segment 7), VP3 (segment 3), NS1 (segment 6) and VP2 (segment 2) genes of an Indian Avikanagar isolate of BTV serotype 1 were amplified using full length and nested primers in cell culture, spiked blood and semen samples (Figs 39-42).

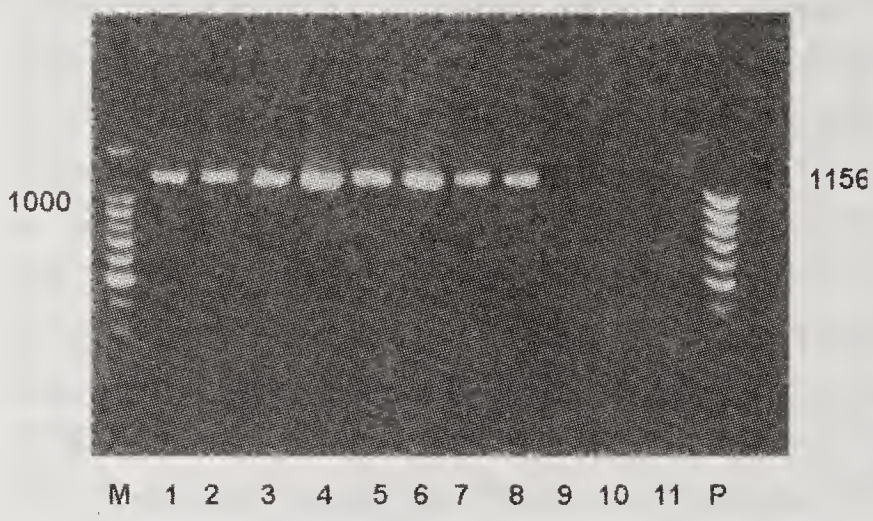


Fig. 39. Full-length amplification of group specific VP7 Gene different isolates of BTV. Lanes 1-8 exhibiting 1156bp PCR product.

In authors' laboratory the sensitivity of the reverse transcriptase-PCR (RT-PCR) for detection of cell culture grown BTV was determined up to 10 infectious virus particles using NS1 gene specific.

RT-PCR is done for RNA viruses. The RNA of BTV is first transcribed into cDNA using reverse transcriptase enzyme of ether avian or murine retrovirus origin and further this cDNA is used for amplification. Different PCR primers used by various workers are listed in Table 26.

Fig. 40. Hemi-nested amplification of VP3 gene. Arrow indicates 380bp PCR product in lane 1.

A number of variations including RT-PCR (Dangler *et al.* 1990), nested PCR (Wilson 1994), multiplex-PCR (Wilson and Chase 1993), immuno-PCR, random primer PCR, *in situ* PCR (McColl and Gould 1991), PCR -ELOSA (MacLachlan *et al.* 1994) in basic protocol have been developed in the past 10 years.

Nested PCR procedure uses two sets of primers in two sequential amplification reactions. The first primer pair amplifies through 25-30 cycles a DNA product, which is then used as template for second set of reaction.

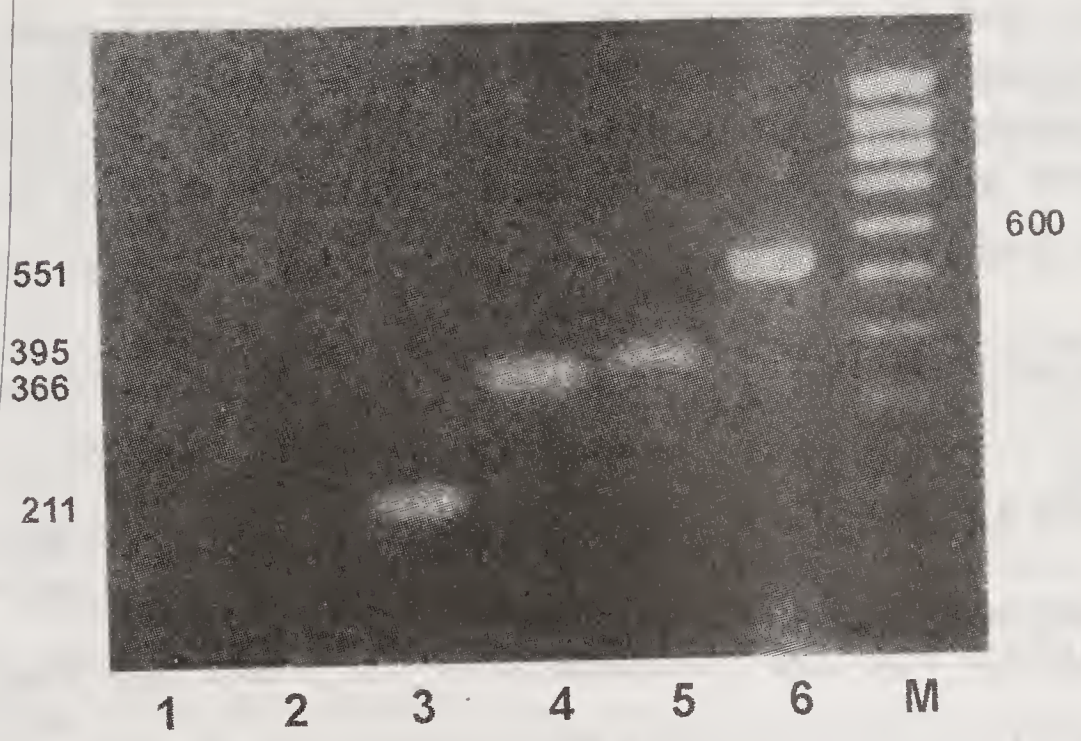


Fig. 41. Amplification of NS1 gene using different primers results in different size PCR products

The primers for the second amplification are located within the first amplified region known as nested-PCR or one is the same as outer primer and one is located within the amplified region is different known as semi- nested PCR. Nested and semi-nested PCR provides greater specificity because it involves 2 rounds of amplification. The second round primers check the specificity of first primer product. This process provides an additional specificity to the reaction and greatly enhances the efficiency of amplification. This nested-PCR test (Fig. 43) was very sensitive detecting an equivalent to 1 plaque-forming unit of BTV viral RNA extracted from infected biting midges (Wilson and Chase 1993).

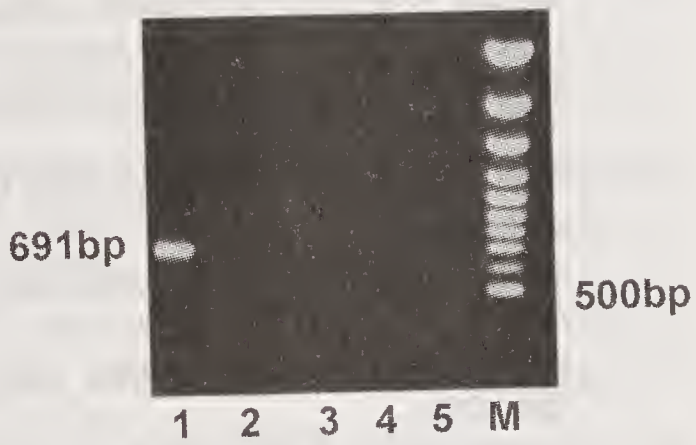


Fig. 42. VP2 amplification. Lane one indicates amplification of 691bp VP2 gene specific PCR product while lanes 2, 3, 4 and 5 are negative for the virus.

In situ PCR has 2 main advantages over conventional PCR—first, it is possible to correlate PCR results with specific cell type in tissue i.e. the cells with positive signal in a tissue specimen can be

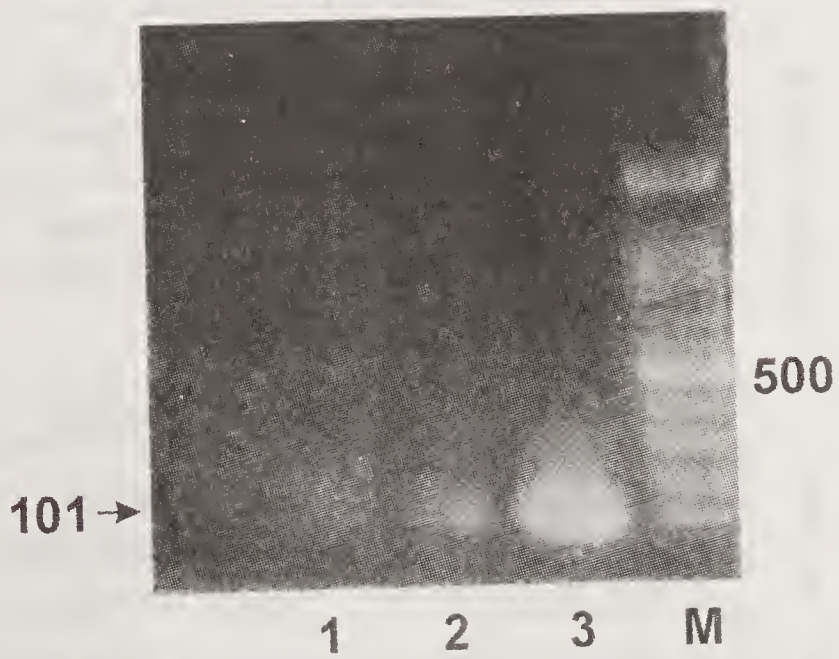


Fig. 43. Nested-PCR of NS1 gene. Arrow indicates 101 bp nested PCR product in lane 3.

identified, and second, there are no chances for contamination by carry-over products. By *in situ* PCR the virus specific sequences can be amplified in morphologically intact cells or tissues. The basic procedure involves application of PCR reagents on fixed cells/tissue sections mounted on

Table 26. Different primers used for amplification of VP2, VP3, NS1 and VP7 genes of BTV

Sr. No.	Sequence 5'-3'	Position	Reference
	VP2		
1	5'-GTT AAA ATA GTG TCG CGA TGG ATG AG-3'	1-26	Wade-Evans *et al.* (1990)
2	5'-GTA AGT ATG ATA GCG CGC GGA-3'	2943-2923	
3	5'-AAT TCC ACG CCG TTG CAA GAT-3'	1844-1813	Designed by the authors
4	5'-ATG GTC GAG TTA ACC TGT TTG ATT ATG TC-3'	1240-1271	
5	5'-TAC CTC TGT TCT TTC-3'	2242-2256	McColl and Gold (1991)
6	5'-TGA TAG CGC GCG GAC CCA CGG TCG ACC GGG TCA TCT CGA GAG AAG TTT TG-3'	2933-2884	
7	5'-TTG TCC ACG CCG AGC GCG CA-3'	604-623	McColl and Gold (1991)
8	5'-ATC GAA CAG GTT CAC TCG GC-3'	1262-1243	
	VP3 gene		
1	5'-CCT GAT GTT TCC AGG ACA AAT TAT ACT C	1055-1082 (1994)	McColl and Gold
2	5'-CCG ATT AAA GGC AAA CCA AAG CGA TAT CC	1434-1410	
3	5' to 3' TAT GTA ACG CTG AGC ATG TAC GTA G (nested primer to be used with primer 1)		McColl and Gold (1991)
	NS1 gene		
1	GTT CTC TAG TTG GCA ACC ACC	11-31	Katz *et al.* (1993)
2	AAG CCA GAC TGT TTC CCG AT	284-265	
3	GTT GGC AAC CAC CAA ACA TGG	19-39	
4	ATC ATC AGC TGC ATC TGG GTT	569-549	Designed by the authors
5	GCA TTT TGA GAG AGC GAT GAT CGC	174-197	
6	TCC CAC TTT TGC GGT AAT CCT CAA	384-361	
7	GTT GGC AAC CAC CAA ACA TGG	19-39	
8	TCC CAC TTT TGC GGT AAT CCT CAA	384-361	
9	GCA TTT TGA GAG AGC GAT GAT CGC	174-197	
10	ATC ATC AGC TGC ATC TGG GTT	569-549	
	VP7 gene		
1	5'-ATG GAC ACT ATC GCA GCA AGA-3'	18-38)	Designed by the authors
2	5'-GTA AGT GTA ATC TAA GAG ACG-3'	1156-1136	
3	5'-GTT AAA AAT CTA TAG AGA TGG AC-3'	1-23	
4	5'-ACA ACT GAT GCT GCG AAT GA-3'	321-340	Tiwari *et al* (2000)
5	5'-AAC CCA CAC CCG TGC TAA GTG G-3'	1090-1069	
6	5'-TCA TTC GCA GCA TCA GTT GT -3'	340-321	

microscopic slides and then the amplification is done as for standard PCR. The amplified product can be detected by using target DNA specific radio active or non-radio active probes by *in situ* hybridisation assay. This procedure has been successfully used for quick detection of BTV in peripheral blood leukocytes of the infected animals.

Multiplex-PCR is based on the basic principle of PCR. In this method, two or more primers pairs sets specific for different target sequences are included in the same amplification reaction. Co-amplification of several target is more cost effective where multiple serotype of a pathogen or different pathogens are possible etiological agents. In this procedure, a large number of samples can be subjected to PCR in a specifically designed plate. Using the multiplex-PCR product detection kits, the result of the amplification can be read directly after completion of the PCR. In this process, running of gel for detection is not necessary. Hence, multiplex-PCR appears to be very useful in processing a large number of samples at a time and can be used for detection of multiple pathogens. Multiplex-PCR is being used in epidemiological investigations for a variety of bacterial and viral pathogens. The availability of commercial kits for conducting multiplex PCR will expand its application in future. The sensitivity of multiplex-PCR is less sensitive then nested-PCR but sufficient to detect virus in field samples (Wilson and Chase 1993). Johnson *et al.* (2000) confirmed the validation of multiplex RT-PCR for serotype determination of United States isolates of BTV. They developed a single tube mutiplex RT-PCR assay. The determination of serotype was based on the size of the relevant amplified product. Procedure was evaluated using all 24 serotypes of BTV and 9 serotypes of epizootic haemorrhagic disease virus. Multiplex PCR detected all the 5 serotypes of BTV circulating in United States. This assay was also compared with virus neutralisation assay. Multiplex RT-PCR assay was more reliable and specific than virus neutralisation. Wade Evans *et al.* (1990) successfully used PCR to detect BTV in blood samples of cattle experimentally infected with BTV. PCR reproducibility allowed detection of as few as 6 molecules of ds RNA, after 60 reaction cycles. Binns *et al.* (2001) reported use of duplex RT-PCR in diagnosis of clinical case of BTV and compared with conventional methods. Assay based on highly conserved S10 genome segment of BTV was developed. The assay detected 5-cell culture infectious doses ($CCID_{50}$) units. The duplex RT-PCR assay was more suitable than virus isolation in embryonated chicken eggs and cell culture assays.

It must not be forgotten that PCR technology amplifies the target sequences, in itself it is not a diagnostic test whereas specific detection of the amplified product using a number of methods make the PCR procedure complete. Products of PCR amplification are usually detected by agarose gel electrophoresis with ethidium bromide staining. To circumvent this cumbersome step of staining and electrophoresis step, colorimetric and chemiluminescence methods have been developed. Although the approaches to visualize DNA products are diverse, most are based on enzyme-linked

assays using biotinylated or digoxigenin labeled primers. The enzyme linked oligonucleotide sorbent assay (ELOSA) for the detection of BTV infection for example, relies on annealing of separate biotinylated and fluorescenated probes to the amplified viral nucleic acid; these complexes are captured on streptavidine coated microtitre wells and detected using a horse radish peroxidase labeled antifluorescene antibody conjugate (MacLachlan *et al.* 1994). PCR holds wider implications in molecular biology. In the specimens when small number of viral particles is suspected, PCR could be used to amplify the viral nucleic acid (Gould *et al.* 1989, Dangler *et al.* 1990).

OIE recommended diagnostic procedures

The following tests/procedures for diagnosis and certification of animals and their germplasm have been recommended by OIE (2004):

Virus isolation: Intravenous inoculation of embryonated chicken eggs (ECE) is the most sensitive technique for isolation of BTV that has been recommended by OIE.

Virus identification: For identification of viruses, following tests have been recommended.

Antigen antibody based tests: Antigen capture ELISA immuno-fluorescence tests utilizing group specific monoclonal antibodies.

Serotyping: Neutralization test is biologically relevant and a number of formats such as plaque reduction assay and microtitre neutralization have been developed. However, there are some problems of cross-reaction.

Nucleic acid based test: Group specific RT-PCR targeting NS1 gene.

Serological tests: AGID assay is easy and cheap to perform. However, it lacks sensitivity and has cross-reactivity with EHDV. Therefore, cELISA is the standard test.

New procedures suggested by OIE for future development and evaluation:

a. Typing by type specific primers and nucleotide sequence analysis
b. Real time PCR
c. IgM ELISA

Molecular understanding of the components of the virus and subsequent expression of the relevant genes in baculovirus and yeast expression systems has allowed production of recombinant reagents for developing more precise and sensitive diagnostic tests. The enzyme immunoassays based on monoclonal antibodies and recombinant antigens have been helpful in distinguishing BTV from other Orbiviruses. Nucleic acid based tests have also been used under laboratory conditions and their application in field surveys still remains unavailable. However, use of PCR in detection of the carrier/reservoir status of the animals and testing of semen and embryos appears quite promising. Though great strides have been made in diagnostic technology, further refinement on the already available diagnostic tools is required.

Agar gel immuno-diffusion test, which has been used for detection of BTV antibodies throughout the world, is less specific and lacks sensitivity. AGID has been OIE recommended test for the screening of ruminants for

international trade purposes. However, now with the availability of other more sensitive and specific tests such as cELISA and RT-PCR, OIE has recommended use of these tests for certification of animals for international trade and quarantine purposes. Recent research on development of PCR based protocols for typing of new isolates has shown potential to replace serum neutralization based serotyping. However, extensive validation of the type specific primers is warranted using BTV isolates from different regions of the world. PCR based typing protocols need to be validated using serum neutralization as gold standard. Since IgM is the first antibody produced in response to infection, detection of BTV-specific IgM in the ruminant animals would indicate recent infection and possibly viriemia. Therefore, a highly sensitive IgM-based ELISA is to be developed and validated internationally. If IgM ELISA is capable of co-relating with viriemia, it could be simple test and have potential application certification of animals for trade. To have an effective vaccination programme, it is important that a highly sensitive and specific test is made available that can differentiate between vaccine strain and wild type BTV. PCR based technology is to be developed for differentiation between wild type and vaccine strains of BTV.

Currently research is also focused on real time PCR which is more expensive and rapid than traditional PCR. Real time PCR is less prone to contamination problem. There are possibilities for development of multiplex PCR also. Most of the recent diagnostic technologies have been developed in advanced countries and are quite expensive. The reagents for PCR and monoclonal antibody based cELISA kits are costly. Hence, indigenization of these technologies is very important.

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12

Vaccines

Vaccination is one of the most effective methods for control and management of any infectious disease. Protective immunisation against BT has been a difficult goal for past several decades. The antigenic diversity exhibited by the known 24 serotypes of BTV and insect vector-borne nature of the disease have been the major impediments towards development of an effective broad based vaccine and control of the disease. Traditional methods for development of an efficacious BTV vaccine have enabled development of live attenuated and inactivated vaccines. However, these vaccines have not found wide applications due to the reasons discussed elsewhere in this chapter. An ideal BTV vaccine should have following features: (i) It should protect against all the serotypes of BTV present in a geographical region; (ii) safe for use in all the susceptible animals including pregnant ones; (iii) no risk for reverting back to virulence; (iv) permits the discrimination of vaccinated from the naturally infected animals; and (v) production of economical and room temperature stable vaccines.

The outer shell proteins, VP2 and VP5 of BTV induce neutralising antibody response and are associated with serotype specificity while inner shell peptides, and VP3 and VP7 are highly conserved in all the serotypes of the virus, hence elicit group specific antibody response. Since VP2 and VP5 induce virus neutralising immune response, these have been targets for development of recombinant vaccines. The conserved proteins VP3, VP7 and NS1 and their corresponding RNA segments have been targeted for development of various immunological and nucleic acid based diagnostic reagents.

Advances in molecular biology, immunology and virology have provided unprecedented opportunities to develop innovative biotechnological approaches using molecular and immunological techniques such as recombinant DNA and hybridoma for producing more effective, economical, thermostable and safer vaccines. This chapter is aimed at providing comprehensive appraisal of current status of BTV vaccines and problems associated with existing conventional vaccines.

Conventional vaccines

Attenuated vaccines: Since BT was first discovered in Africa, most of the earlier efforts for development of BTV vaccines have been made in this continent. The South African scientists have pioneered the development and

production of attenuated live BTV vaccines because of their historic experience with BT through several decades. In earlier studies the field viruses were passaged up to 80-times in developing chicken embryos. However, recent work has suggested that 20-50 passages either in embryonated chicken eggs or BHK-21 cell line is adequate to render the virus avirulent (Johnson *et al.* 1992). The attenuation process selects viruses that adapt to grow *in vitro* and have decreased ability to grow in sheep and cause clinical disease. Lower passage level viruses induce strong immune response but retain cability to cause clinical manifestation where as too many passages *in vitro* will generate viruses that will not result in clinical disease and induce poor protective immune response (Murray and Eaton 1996).

Both monovalent and polyvalent avirulent live vaccines have been developed by serially passaging the virulent BTV through embryonated chicken eggs and BHK-21 cell culture. Live attenuated vaccines are very effective in controlling the clinical outbreaks of BT in endemic areas (Erasmus 1980) and in face of outbreaks (Mccowan *et al.* 1956, Mckercher *et al.* 1957). Only one dose is sufficient to induce protective immune response against homologous challenge. The attenuated BTV vaccines thus produced have been extensively used in Africa and Middle East resulting in dramatic reduction in economic losses due to BT. Presently in South Africa, sheep are vaccinated with 3 pentavalent attenuated BTV vaccines at 3 weeks intervals. The sheep immunised thus, are exposed to 15 live serotypes of BTV in 6 weeks (Schultz and Grieder 1987).

The attenuated BT vaccines suffer from several drawbacks as compared to inactivated alternatives. Firstly, attenuated strains of Australian serotypes have been shown to be teratogenic (Erasmus 1990, Johnson *et al.* 1992, Johnson and Roy 1996). Secondly following vaccination and during viraemia, attenuated viruses may appear in semen of vaccinated bulls and rams. Thirdly, the attenuated virus strains could be transmitted by *Culicoides* midges as natural viruses. Homologus and heterologus serotypes readily reassort and the progeny viruses may contain random combination of parentral genes (Sugiyama *et al.* 1982, de-Mattos *et al.* 1991). Thus the ability of BTV to mutate or reassort *in vitro* and *in vivo* conditions, argues against use of live vaccines, especially in combination of multiple serotypes. The release of attenuated viruses in the environment may also lead to reversion of virulence. In USA an attenuated vaccine against BTV serotype 10 is commercially available. However, limited demand by the sheep farmers, was disincentive to vaccine manufactures to produce attenuated vaccines against other commonly encountered BTV serotypes in USA. Additionally the investment needed by the vaccine manufactures to meet the safety, potency and efficacy requirement for federal licensing was considered to be too high for an obsolete vaccine technology (Strating 1985). These considerations have been major deterrents in development and commercial production of attenuated vaccines in USA.

In Australia, a BHK-21 cell culture attenuated vaccine strain of Australian BTV1 was evaluated for its protective efficacy (Johnson *et al.*

1992). The vaccine strain was found teratogenic when administered in first trimester of pregnancy. Bluetongue virus specific antibodies were detected in the sera of precolostral lambs indicating active immunisation of lambs if the ewes were inoculated with the attenuated vaccine at day 40 of gestation. The current BTV vaccine strategies in Australia have been recently reviewed by Murray and Eaton (1996). The studies conducted in Australia have demonstrated that the attenuated virus strains which elicit only a mild febrile reaction, generate a titre of less than 10^3 pfu/ml in the blood at the time of the height of viraemia and elicit neutralizing antibody response are selected for vaccine development. It is believed that viraemia lower than 10^3 pfu/ ml will ensure that the viruses will not be transmitted by blood feeding insects (Gard *et al.* 1995). The studies conducted in our laboratory using BHK-21 adapted attenuated local isolate of BTV 1 suggested that the virus produced very mild clinical signs in crossbred sheep and elicited neutralising antibodies which persisted at least 60 days (Chander *et al.* 1991). Bluetongue is widely prevalent in India, no vaccine is available here and the practice of vaccination is not being followed mainly due to multiplicity of serotypes and cost of the imported attenuated vaccine. Moreover, the attenuated vaccines currently available suffer from several drawbacks described earlier.

Inactivated vaccines: Unlike attenuated virus vaccines that replicate in vaccinated animals and produce large amount of antigen *in vivo*, the only antigenic stimulus received with killed virus is derived from the vaccine itself. Hence, the dose of inactivated virus has to be much higher and perhaps as higher as 100-times than the live virus vaccines. The use of adjuvant is necessary in inactivated vaccines. Viruses have been inactivated by a number of agents including beta propiolactone (BPL) (Parker *et al.* 1975), binary ethylenimine (BEI) (Stott *et al.* 1979) or gamma irradiation (Barber and Cambell 1984). Parker *et al.* (1975) reported that neutralizing immune response persisted for one year after immunization with BPL-inactivated virus in an oil-based adjuvant. However, they could not conduct challenge experiment. Similarly neutralizing antibody response was detected in sheep immunized with gamma irradiated virus (Barber and Cambell 1984). However, neutralizing antibody response was not detected in the animals vaccinated with BEI inactivated virus (Stott *et al.* 1979). The inactivated BTV vaccines have been reported to have variable degree of protection ability. Stott *et al.* (1979) observed that sheep inoculated with inactivated BTV vaccine developed precipitating but not neutralising antibodies. However, when vaccinated sheep were challenged after 6 weeks of vaccination, some sheep were protected probably due to cell-mediated immunity. It was not clear why only some sheep were protected. Berry *et al.* (1981) indicated that there were pronounced breed variations in immune response to inactivated vaccines in sheep. Tembhurne (2000) and Ramakrishnan (2002) also evaluated the inactivated vaccines against BTV and reported that inactivated vaccine produced neutralizing antibodies in immunized animals.

The inactivated vaccines may have a number of advantages including no risk for transmission by *Culicoides vector,* transmission of the virus to the foetus and absolutely no possibility of reverting back to the virulent form. Though the inactivated vaccines do not cause clinical disease or viremia, they suffer from other drawbacks such as repeated injections, poor immune response and side effects due inactivating agents. Despite several advantages over the attenuated live vaccines, the inactivated BTV vaccines have not been economically attractive enough to the sheep farmers in the USA. Probably the main reason for the failure to develop inactivated vaccines was recognition of the consequences of the incomplete virus inactivation in the vaccine preparation. In contrast safety issue surrounding attenuated BTV vaccine is relatively recent. In addition, in endemic BTV for example South Africa, where 21 serotypes circulate, the impact of an addition circulating attenuated virus arising from live vaccine does not substantially add to the problem (Gard *et al.* 1995).

Molecular approaches to BTV vaccines

Owing to the inherent problems associated with the conventional attenuated and inactivated BTV vaccines, alternative approaches employing biotechnological techniques are being explored.

Recombinant peptide vaccines: VP2 and VP5 together generate neutralising immune response. This has raised the possibility of using recombinant immunogens as vaccine against BTV. Both of these proteins have been expressed in prokaryotic and eukaryotic expression hosts applying recombinant DNA technology. Huismans *et al.* (1987) demonstrated that purified VP2 induces protective immune response against BTV. Subsequently Roy *et al.* (1990) reported that the VP2 obtained through recombinant baculovirus also elicited immune response that protected the immunised sheep against homologous virulent virus challenge. They further reported that combination of VP2 and VP5 produced better neutralising immune response which conferred protection. This is hypothesised to be due to synergistic effect of two proteins which probably offers better conformational representation of the neutralising epitopes. The purified VP2 and VP5, either produced traditionally or through recombinant DNA technology, produce protective immune response which is short lived. Thus peptide vaccines have limited utility and are not economically attractive vaccine to manufacturers.

Recombinant virus-like particles (VLPs) vaccine: To develop better recombinant immunogens for immunisation against BTV, Roy *et al.* (1992) have developed an innovative approach to produce BTV-like particles (VLPs) without the viral genome using dual baculovirus expression system. The genes corresponding to outer capsid proteins (VP2 and VP5) and major core proteins (VP3 and VP7) have been cloned in two separate baculovirus expression vectors. The simultaneous co-infection of the *Spodoptera frugiperda* cells with both the recombinant baculoviruses produced the VLPs not morphologically distinguishable from the conventionally cell culture

produced authentic BTV particles. The small scale studies conducted in sheep in South Africa and Australia using the BTV-VLPs as candidate vaccine, have clearly suggested that the VPLs when used in combination with appropriate adjuvant elicit protective immune response and the vaccinated animals resist the homologous virulent virus challenge (Johnson and Roy 1996). The VLPs lack the genetic material hence these can not cause infection to the vaccinated animals and can not pass on to the foetus. Since these are non-infectious, there is no risk of reverting back to virulent form and transmission by the vector. Another advantage of VLPs is that a combination of different serotypes can be made in the form of cocktail which protects against a number of serotypes prevalent in a particular geographical region. One can visualise the potential of the technology to produce vaccine chimeras representing different serotypes (Roy 1996). Another possibility of making hybrid VP2 and VP5 representing the immunogenic epitopes of different serotypes also remains workable alternative.

Recombinant core-like particle (CLPs) vaccine: The BTV core-like particles can be synthesised in *S. frusiperda* insect cells infected with recombinant baculovirus carrying genes encoding for VP3 and VP7 group specific proteins of BTV. These two proteins when produced in *S. frusiperda* insect cells, assemble to form BTV core-like particles without the viral genome (French and Roy 1990). Recent preliminary studies carried out in Merino sheep in Australia using recombinant CLPs along with adjuvant have suggested that the immunised sheep partially resists the virulent virus challenge (Hosseini *et al*. 1996). Unlike VLPs the partial protection induced by CLPs is not serotype restricted. This makes CLPs very attractive vaccine candidate because the protection is broad based possibly due to cell-mediated immune response.

The CLPs have been proposed as potential broad-spectrum BTV vaccine. It has been suggested that in outbreak of BTV infection, where serotype specific vaccines are not available or the serotype is unknown, CLPs could be used to reduce the impact of the infection (Hosseini *et al*. 1996). Since these particles are non-infectious and devoid of genetic material, these do not have the risks associated with live attenuated vaccines. More over, CLPs can be engineered to distinguish between vaccinated and BTV infected sheep by developing appropriate ELISA.

Vectored vaccines: Protective immunogens (VP2 and VP5) of BTV have also been cloned in expression systems like capripox and vaccinia viruses (D.N.Black, personal communication, UK). However, field trials using such recombinants are yet to be reported.

Bluetongue has achieved global dimensions causing economic losses of alarming proportions. The major problems in its control are multiplicity of serotypes and different species of the vector involved in the transmission of BTV in different parts of the world. However, the EEC countries have been able to significantly control the disease due to vaccination and strict quarantine measures. Australia and Canada are also taking effective safety and quarantine measure to contain the disease. Preliminary results obtained

through immunisation of sheep with genetically engineered VLPs and CLPs clearly indicate future prospects for developing better, safer and potent vaccines. Chimeric viruses-like particles produced by baculovirus expression system and live vectored vaccines using vaccinia and capripox, hold considerable promise for developing future BTV vaccines. The comparative

Table 27. Comparative features of bluetongue virus vaccines

Feature	Type of vaccines		
	Attenuated	Recombinant	Killed/ inactivated
Safety	Major concern	Safe	Reasonably safe
Availability and experience	Extensive	None	None
Experimental efficacy	Very good	Good evidence	Limited
Relative cost	Inexpensive	Expensive	Expensive
Doses required	One shot	Multiple shots	Multiple shots
Duration of immunity	One year	Not known	Six months

features of BTV vaccines are summarized in the Table 27.

Molecular characterization of all the vaccines strains is urgently required. The sequence data of all the vaccines should be deposited in the Genbank made available globally through FAO/OIE. Similarly technologies that distinguish between vaccinated animals and naturally infected animals need to be developed and validated. Though great progress has been made in understanding the molecular biology and immunobiology of BTV, the livestock farmers are yet to enjoy the fruits of new biotechnology. However, due to high cost and multiplicity of serotypes, subunit vaccines are unlikely to be available to the livestock farmers in near future.

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Control Strategies

Control of insect transmitted diseases is a very challenging task. Bluetongue is an insect transmitted and multi-host disease involving domestic and wild ruminants. Therefore, the control of BT becomes further complicated. Currently, two approaches are being pursued for its control with considerable success in minimising the economic losses. First and the most practicable way has been the protection of susceptible animals against the disease by immunoprophylaxis (vaccination) and the second involves control of the *Culicoides* vectors responsible for its transmission. Different serotypes/strains of BTV cause variable severity of disease in different breeds/species of animals. Therefore, breeding of animals that are relatively resistant to BT disease could minimise economic loss resulting from morbidity and mortality. Present status of both strategies is presented here.

Immunoprophylaxis

Progress in means of immunisation against BT disease has tremendous voids. Way back in 1905, Spreull devised a method for immunisation of sheep. His procedure consisted of inoculating virulent blood virus either simultaneously with immune serum or delaying the serum injection for several days. Subsequently, Theiler (1908) discovered that after a certain strain of BTV passaged 10-times in susceptible sheep, it had completely lost its virulence. Although there were problems with the use of vaccine, vaccination against BT with it continued in South Africa for 40 years, and 50 million doses were sold (Neitz 1948). Alexander *et al.* (1947) were probably the first group of workers who had adapted BTV in embryonating chicken eggs. This finding was a fortuitous breakthrough and allowed various strains of the virus to be attenuated since no cell cultures were available at that time. This paved the way for development of a polyvalent vaccine. Subsequently, animal cell culture technique was developed and Haig *et al.* (1956) adapted several egg attenuated viruses in tissue culture and making it possible to perform neutralisation assay *in vitro*.

In 1963, Howell observed that there were several breakdown of polyvalent vaccines. Therefore, South Africa evaluated a polyvalent vaccination strategy that immunised sheep against 15 serotypes which are prevalent in that country. Subsequently, USA adopted the South African pattern of vaccines. Egg adapted vaccines produced viraemia in sheep which was of sufficient titre to infect vectors feeding on the vaccinated animals at

the height of the febrile response. An egg adapted commercial vaccine was capable of killing young white-tailed deer if administered as recommended by the manufacturer. Therefore, there was a view that live modified vaccines should not be used for prophylaxis against diseases transmitted by insect vectors unless the viral concentration of the blood after vaccination is low enough to preclude infecting haemophagous insects. This dictum would hold true even for so called attenuated vaccines since little is known concerning the possibility of revitalisation of the attenuated virus by serial passage between host animal and the insect vector. Therefore, the safest vaccines for the virus diseases transmitted by insect vectors would appear to be inactivated or killed vaccines. It is usually more difficult to stimulate a satisfactory immune response with a killed than that with living viral vaccines, especially when a polyvalent resistance is required. The problem is further complicated by the fact that the arboviruses are prone to undergo antigenic drift. This is especially true for BTV as attested by the fact that there are 24 distinct serotypes of the virus. The development of an effective vaccine for sheep, cattle, goat and wild ruminants kept in zoos would require a great deal of cerebration and concerted research efforts. The use of such vaccine in cattle is doubly complex since the disease may be inapparent in majority of infected cattle. The available information suggests that vaccination with commercial attenuated vaccine may not be practical in areas other than those in which BT virus is enzootic.

Control of the vector

Several approaches have been tried in search of an effective way to control *Culicoides* vector. Conventional method to control the vector has been the use of chemical insecticides spray in livestock housing places. Another approach which has been tried, involves application of insecticides on the skin of animals during vector season e.g. monsoon. Dipping of sheep with insecticides has been in practice in several countries as an effective method for control of ectoparasites. Similarly, parenteral administration of certain insecticides has also been reported to reduce incidence of BT. In the authors' experience, administration of ivermectin in sheep greatly reduced prevalence of BTV in an organised sheep breeding farm near Hisar. Ivermectin also reduces incidence of ectoparasites. Though synthetic chemical insecticides have been very effective in destroying the *Culicoides* and their larvae, these pose a great risk to human and animal health and environment. Further selected mortality of more susceptible genotypes of the vector following repeated application of insecticides has resulted in rapid development of resistance. Consequently, the last half of this century has seen efforts to develop alternative pest management strategies that are non polluting and environmentally benign. One such strategy that has attracted much interest involves use of naturally occurring bioinsecticides such as products from trees like *Neem* and micro-organisms like *Bacillus thuringiensis* (Bt). The research and development of eco-friendly bioinsecticides has been on focus due to increasing environmental concerns.

Another approach to manage *Culicoides* midges is by creating hygienic conditions in the places where the susceptible animals are housed. Breeding habitats of *Culicoides* midges have been thoroughly investigated. The vector breeds in stagnant water, edges of the running water channel, cow dung, muddy and marshy areas as have been described in detail under vector biology chapter in the book. The destruction of these habitats eliminates breeding sites of the vectors. *Culicoides* midges breed and develop in soft, silty mud area water and mud areas exposed to direct sun light. The larvae develop in shallow water and mud at the edge of such areas, therefore, muddy water around watering troughs should be eliminated. The edges of overflow ponds and the banks of irrigation ditches should be kept relatively steep to eliminate shallow mud areas, and seepage from ditches should be eliminated. Usually overflows for irrigation , unless permanent, do not create ideal breeding sites, in fact fluctuating irrigation reservoir will decrease the possibility of breeding sites, which develop along the shallow edges, either by flooding or drying out of ideal breeding sites.

Howell (1963) has reported from South Africa that vectors prefer to remain in low lying-pastures and *Culicoides* species are, for the most part , twilight feeders. These circumstances have also been observed in relation to epizootics of the USA, therefore, animals should be moved to high pastures during the evening hours. Spraying of animals with insect repellent is helpful in areas where the insect vectors are particularly numerous. The insect repellents that are incorporated in the feed may be developed and perhaps the genetic make-up of the insect vectors may be altered to the point where susceptible vectors do become or can be rendered non- susceptible. Another area which has been gaining importance is genetic manipulation of male population of vectors to make them sterile. Jones and Foster (1974) demonstrated that the lines of *C. variipennis* resistant to BTV can be developed in the laboratory. The control of the disease may become possible by lowering the vector capacity of the vector species without controlling the species itself. Genetic manipulation approach appears to have potential for eco-friendly way to control vector population.

Quarantine

Quarantine involves isolation of animals that are either infected or suspected to be infected or non-infected animals which are at the risk. This method is of importance for the countries importing animals from countries where BT is endemic. Animals under quarantine should be properly checked using the diagnostic assays to confirm the disease free status of the animals.

Quarantine as well as slaughter of BT affected animals has been used by several European countries where it is not enzootic. In the past over 10 years, China has also used slaughter of affected animals as one of the components of control strategy along with vaccination and application of insecticides. Preventing and controlling the spread of BT from outbreak sites involve quarantine measures, isolation of sick animals, quarantine of epidemic area, insect destruction on grassland where the epidemic has

occurred and surrounding areas, and slaughtering the sick and suspect animals.

Combination of all the approaches appears to offer a sound way of developing BT control programme based on the geographic regions. Ecological consequences should also be kept in mind while selecting the control strategies for BT. It has been argued that sometimes during control programme of an infectious diesease, the balance of nature in an ecosystem disturbs. As sometimes use of insecticides for controlling the *Culicoides* vector might result in the large scale mortalities of birds and other mammals that might have ingested the insecticides. Thus all care should be taken while going for the use of vector control methods.

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